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World Journal of Gastroenterology

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World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1353 experts in gastroenterology and hepatology from 68 countries.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Natural history of hepatic metastases from colorectal cancer - pathobiological pathways with clinical significance

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Abstract

Colorectal cancer hepatic metastases represent the final stage of a multi-step biological process. This process starts with a series of mutations in colonic epithelial cells, continues with their detachment from the large intestine, dissemination through the blood and/or lymphatic circulation, attachment to the hepatic sinusoids and interactions with the sinusoidal cells, such as sinusoidal endothelial cells, Kupffer cells, stellate cells and pit cells. The metastatic sequence terminates with colorectal cancer cell invasion, adaptation and colonisation of the hepatic parenchyma. All these events, termed the colorectal cancer invasion-metastasis cascade, include multiple molecular pathways, intercellular interactions and expression of a plethora of chemokines and growth factors, and adhesion molecules, such as the selectins, the integrins or the cadherins, as well as enzymes including matrix metalloproteinases. This review aims to present recent advances that provide insights into these cell-biological events and emphasizes those that may be amenable to therapeutic targeting.

Key words: Colorectal cancer; Metastatic cascade; Liver metastasis; Liver sinusoids; Neovascularization

Core tip: The multi-step process of colorectal hepatic metastases includes numerous molecular pathways and cellular interactions with potential clinical interest. Mutations at the initial site of colorectal carcinogenesis, such as *p53* and *APC*, neoplastic cell interrelationship with the stromal macrophages, neoangiogenesis through growth factors, such as the vascular endothelial growth factor and platelet-derived growth factor, the role of hepatic sinusoidal cells, such as Kupffer cells, the expression of adhesion molecules, including the selectins and the integrins, are all crucial stages/events within the metastatic process. The exploration and analysis of recent research data may contribute to a better understanding of their clinical significance and may lead to new therapeutic strategies.

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INTRODUCTION

Carcinogenesis [carcino (καρκίνος) = cancer, and genesis (γέννεσις) = birth] and metastasis [meta (μετά) = after, next and stasis (στάση) = arrest] are both words of Greek origin, expressing the onset and the advanced progression (spread to another location) of cancer, respectively. Cancer development is a complicated biological process

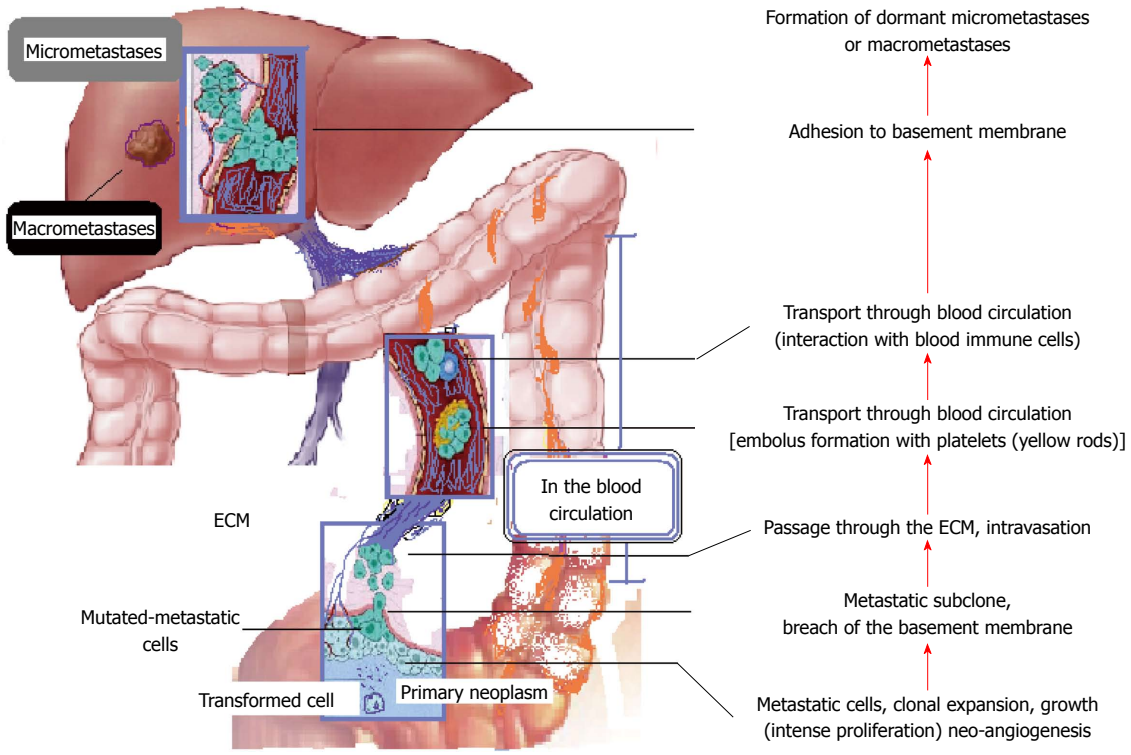

Figure 1 The invasion-metastasis cascade[1,178]. Successive interrelated stages/steps until foreign tissue colonisation and the formation of macrometastases. The paradigm of colorectal liver metastasis, from the large intestine through the superior and inferior mesenteric veins and the portal vein to the liver. ECM: Extra cellular matrix.

that includes numerous poorly understood aspects and attracts the greatest interest of biomedical sciences. It is a succession of interrelated, well-defined stages, usually termed the invasion-metastasis cascade[1,2].

The primary stage in the natural history of carcinogenesis starts with the transformation of normal cells into malignant derivatives at their initial site. Most interestingly, this transformation is also multistep and includes several genetic and phenotypic cellular alterations. Thus, there are two processes that progress in parallel, the metastatic cascade and the cancer cell transformation[3,4].

Primary mutations that signal the initiation of carcinogenesis lead to a progressive cellular proliferation and tumour formation. Neovascularisation (neoangiogenesis) and invasion of normal tissue follow, until several malignant cells are detached from the primary tumour (disaggregation), migrate and intravasate; this is the onset of metastasis. The next steps may be evasion of the immune system, cell survival in the hostile environment of the systemic circulation, arrest of the circulating tumour cells and adhesion to the endothelium lining the capillary bed of the target organ, and extravasation. The final stages of the metastatic cascade are evasion of host defences, establishment of a new blood network to supply the development of a new secondary tumour, and colonisation (Figure 1)[3,5,6].

All these successive stages demand multiple properties from malignant cells, and failure or inadequacy in any of them cancels the entire metastatic cascade. These properties are acquired through multiple mutations. It is uncertain whether cancer cells already possess most of their new potential when they begin the metastatic sequence or if this is gradually acquired. However, the colonising ability is strongly believed to appear later in the tumourigenicity[4,7]. Importantly, various laboratories have confirmed different cell subpopulations in the primary tumour site. Concurrently, the metastatic tumour created in a distant location by cancer cells is considered a different entity from the primary one, because metastatic cells show phenotypical and genetic differences from their ancestors[8-10].

While micrometastases are achieved by several cancer cells, macrometastases rarely occur. It is believed that micrometastases are the final metastatic stage for the vast majority of malignant cells, which never succeed in surviving or adapting in the inhospitable environment of the foreign tissue. In accordance with that belief, cancer patients may present myriad of micrometastases in multiple organs and tissues, although without any clinical evidence[11,12].

This general pattern of carcinogenesis and metastasis applies to most cancer types with certain modifications and adaptations according to implicated cells, tissues and organs[1]. Colorectal hepatic metastasis attracts a particular scientific interest due to the high frequency of colorectal cancer (CRC) (3rd most common cancer in the developed countries and 2nd leading cause of cancer-related mortality) and the unique properties, functions and role of the liver in the human body[13,14]. This review will describe the metastatic journey of CRC cells from the large intestine

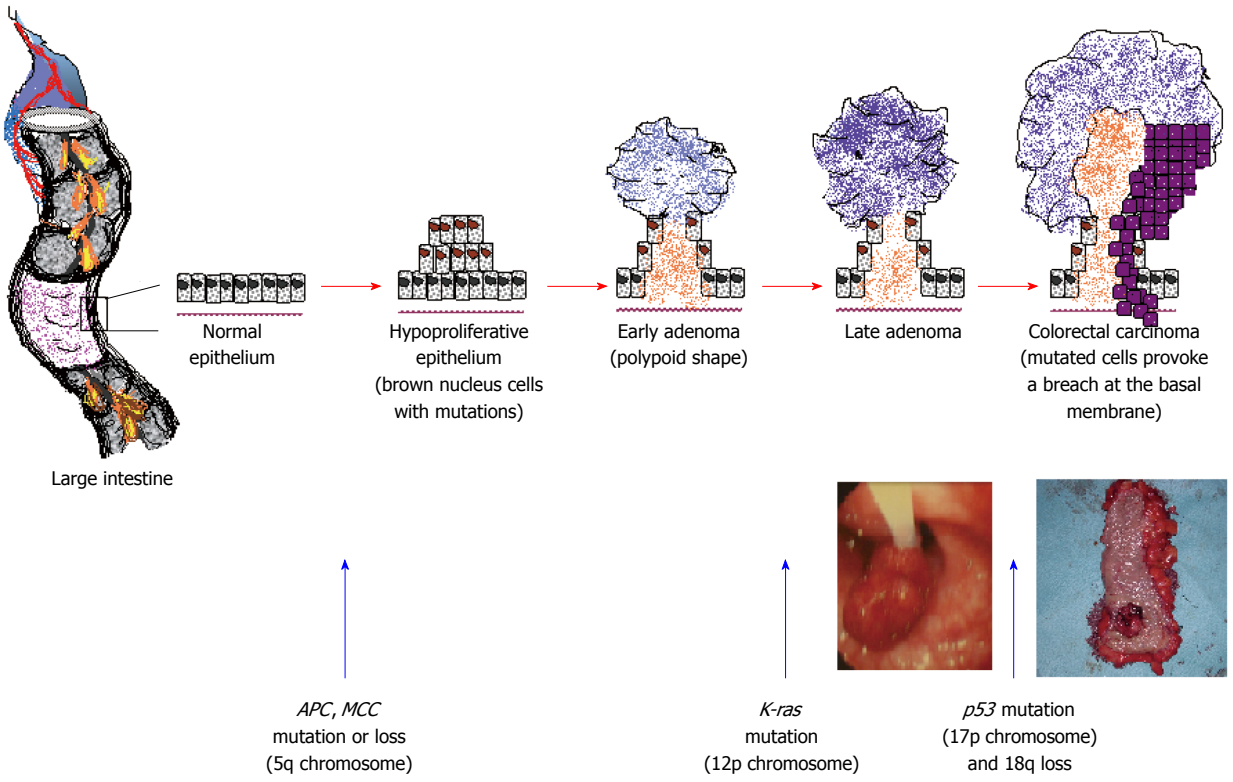

Figure 2 A sequence of mutations for colorectal epithelial cells generates the formation of polypoids, adenomas and finally carcinoma[15,16,19]. Polypoid and carcinoma clinical photos (authors' archives) underneath the figures.

to the liver and will investigate the normal-malignant cell interactions, the role of the tissue microenvironment and the genetic and molecular pathways that mediate this carcinogenic-metastatic process.

INITIAL GENETIC ALTERATIONS OF EPITHELIAL CELLS IN COLORECTAL CARCINOGENESIS

Mutations in CRC include alterations in tumour suppressor genes such as *p53* and *APC*, DNA repair genes such as *hMLH1* and *hMSH2*, and/or oncogenes such as *K-ras* and *c-Myc*. In this way, normal colorectal epithelial cells gain, among other abilities, the capacity of uncontrolled growth and evasion of apoptosis (programmed cellular death). Rapid cell proliferation usually generates the formation of a polypoid structure in the epithelium that may evolve to adenoma, which is a precancerous lesion. An adenoma may progress to CRC, as the mutations accumulate and CRC cells acquire all the necessary properties; single cancer cells or small clusters of them may finally detach from the initial intestinal tumour and migrate (Figure 2)[15,16].

There are at least two well-described genetic pathways that may generate CRC. The most common, which mediates up to 60% of carcinomas, is the chromosomal instability pathway. This is the result of mutations of the *p53*, *APC* and protooncogene *K-ras*, allelic loss of 18q and aneuploidy. The role of the APC gene is crucial in tumourigenesis, as 100% of patients with the familial adenomatous polyposis (FAP) syndrome who carry this mutation develop CRC if they receive no treatment. Another genetic pathway is the microsatellite instability (MIN) one, also termed replication error pathway, which is responsible for approximately 20% of carcinomas. These neoplasms have aberrant DNA mismatch, a near-diploid karyotype, sparse *p53*, *K-ras* and *SMAD4* alterations, but also frequent *BAX*, *BRAF* and *TGF-BIIR* mutations. Hereditary nonpolyposis colon cancer (HNPCC) is attributed to the MIN pathway, accounts for 5%-6% of CRC, and 80% of these patients develop cancer in their lifetime. In HNPCC, MIN is a consequence of mutations in DNA mismatch repair genes (*hMSH2*, *hPMS1*, *hPMS2* and *hMLH1*). Notably, other genetic pathways also exist, such as the cytosine phosphodiester guanosine island methylator phenotype pathway. While in the absence of methylation genes are normally expressed, in the presence of promoter methylation, genes are transcriptionally downregulated. When tumour suppressor genes are involved, carcinogenic mutations occur. A whole subclass of CRC tumours include high proportions of hypermethylated genes[17-19].

INTERACTIONS OF NEOPLASTIC CELLS WITH THE STROMA CELLS AND THE EXTRACELLULAR MATRIX AT THEIR INITIAL SITE - LARGE INTESTINE

The promotion of the initial stages of colorectal carcino-

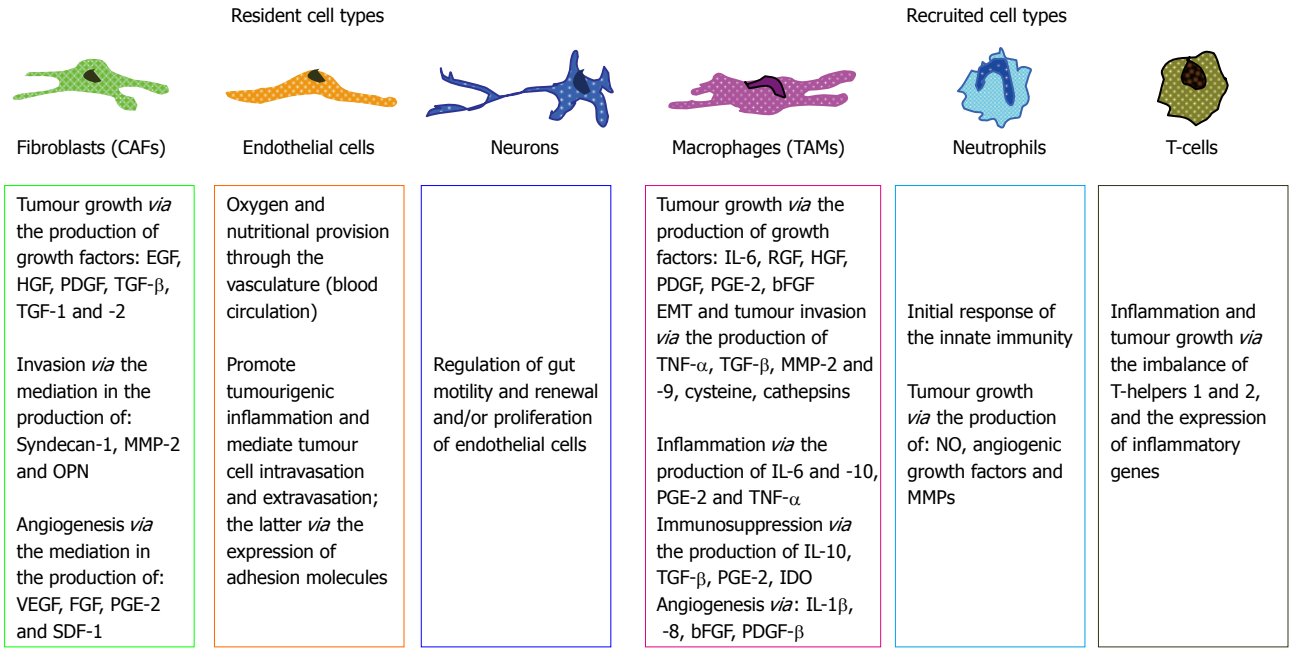

Figure 3 Various cellular types[22,23,112]. Various cellular types (resident: fibroblasts, endothelial cells and neurons, or recruited: macrophages, neutrophils and lymphocytes) which mediate cancer progression and growth in the colorectal microenvironment. bFGF: Basic fibroblast growth factor; CAF: Cancer associated fibroblasts; ECM: Extracellular matrix; EGF: Epidermal growth factor; EMT: Epithelial-to-mesenchymal transition; HGF: Hepatocyte growth factor; IDO: Indoleamine 2,3-dioxygenase; IGF: Insulin growth factor; IL-10: Interleukin 10; MMP: Matrix metalloprotease; NO: Nitric oxide; OPN: Osteopontin; PDGF-β: Platelet-derived growth factor-beta; PGE2: Prostaglandin E2; SDF-1: Stromal cell-derived factor-1; TAM: Tumor-associated macrophages; TGF-β: Transforming growth factor-beta; TNF-α: Tumour necrosis factor-alpha; VEGF: Vascular endothelial growth factor.

genesis depends on the communication and interrelationship of neoplastic cells with the stroma. The term stroma, also referred to as reactive stroma in carcinogenesis, includes the endothelial cells, fibroblasts, immune cells (such as macrophages, lymphocytes and neutrophils), as well as non-cellular elements, such as the extracellular matrix (ECM). It appears that numerous special characteristics of the reactive tumour stroma, concerning cellular, architectural or chemical elements, support a reciprocal tumourigenic communication with neoplastic cells *via* the basement membrane[20]. These characteristics may be a high number of fibroblasts, altered molecular expression on the cellular surface and the cytoplasm of endothelial cells, macrophage recruitment, increased capillary density, ECM rich in fibrin and collagen-1. Furthermore, the production and secretion of a plethora of chemical compounds, including cytokines and growth factors in the colorectal stroma, mediate the promotion of carcinogenesis (Figure 3)[21-23].

Fibroblasts

Fibroblasts within a tumour appear to harbour mutations that transform them into myofibroblasts that are termed cancer-associated fibroblasts (CAFs). Apart from normal fibroblasts, CAFs may also originate from endothelial cells, epithelial cells, preadipocytes and bone marrow-derived progenitors[24,25]. Interestingly, *CAF* mutations may refer to a variety of genes encoding multiple growth factors, cytokines, enzymes and ECM-related proteins. Various studies have shown that CAFs have the potential to produce transforming growth factor beta (TGF-β) in an autocrine or paracrine way, triggering CRC cell detachment from their initial site[26,27]. Moreover, a recent study from Zhu *et al*[28] has demonstrated that TGF-1 may induce plasminogen activator 1 (PAI-1) transcription in CAFs. PAI-1 mediates the fibrinolytic activity in the vasculature, is widely expressed throughout tumours and is associated with malignant invasion and neoangiogenesis[29,30]. Taking together these experimental data, CAFs appear to play an important role in various aspects of carcinogenesis and metastasis, including migration, matrix degradation, invasion and angiogenesis[26,31].

Macrophages

The development of a tumour causes an inflammatory reaction where immune cells may be implicated. Macrophages are potentially the most important tumour-associated immune cells. They may constitute a considerable amount of the initial tumour mass and they correlate with tumour poor prognosis. Although macrophages act as tissue scavengers in general, eliminating any potential harmful element (invading cells or chemicals), cancer cells may use macrophage products in their favour, masking their surface antigens and thus avoiding the tumouricidal action of immune cells. In the invasion-metastasis cascade, macrophages play a significant role in the promotion of inflammation, stroma and ECM remodeling, angiogenesis, neoplastic cell invasion, intravasation and seeding at foreign sites[32-34].

Neoangiogenesis at the initial site of CRC is crucial for tumour development since oxygen diffusion alone from the normal capillary network is unable to supply a

tumour larger than 1-2 mm. Macrophages regulate the critical process of neovascularisation through vascular endothelial growth factor (VEGF) production[35]. VEGF acts directly on endothelial cells promoting their proliferation, migration, invasion and high vascular permeability[36,37]. Another paradigm of the macrophage supporting role for malignant colorectal cells is through the macrophagic removal of apoptotic CRC cells that express sulfoglycolipids SM4. While such a process initially appears to be tumouricidal, the increased secretion of interleukins and TGF-β1 may contribute to tumour development and angiogenesis activation[38].

Lymphocytes

Lymphocytes constitute another immune cell category implicated in tumourigenesis with a favourable prognosis. In advanced CRC, the presence of T lymphocytes favours a better clinical outcome for patients suffering from the disease[39-41]. A recent study by de Miranda *et al*[42] showed that high tumour infiltration by activated $CD8^+$ T cells in patients with Lynch CRC correlated with early staging of the primary tumour and absence of lymphatic metastases. While immune cells mainly support the defending system of normal tissue against neoplastic cells, the latter use genetic and molecular pathways that promote the evasion of immunosurveillance. CRC cells may express the Fas ligand on their surface and bind Fas-expressing immune cells, thus triggering apoptotic mechanisms for the latter[43,44]. An alternative mechanism of escaping immunosurveillance for tumour cells is the high expression of carbohydrates on their cellular membrane. This alters the CRC cell surface antigen profile, impeding their recognition and destruction by immune cells[45]. Furthermore, cells of normal colorectal tissue may also support neoplastic cells in evading immunosurveillance. Tumour-associated macrophages are able to produce arginine metabolites, which cause T-lymphocyte death. Similarly, in the lymph nodes of advanced neoplasms, dendritic cells play an immunosuppressive role rather than an immunoreactive one. Thus, instead of activating T-lymphocytes, they induce tumour tolerance[46,47].

ECM

ECM supports the cells mechanically and assists their physicochemical functions through the provision of pathways for migration and the maintenance of growth factors. Adhesion molecules play a critical role in the ECM-cell connection, with the integrins being particularly important. The ECM consists of 5 classes of molecules (collagens, fibronectins, glycans, hyaluronans, laminins and proteoglycans). Every class includes multiple isoforms, which are cell-dependent. ECM composition and structure vary with developmental stage, tissue and disease[48,49].

The basement membrane (BM) or basal lamina is a specific ECM-type meshwork produced by epithelial and stromal cells, and it plays a crucial role in carcinogenesis and metastasis[50,51]. BM chemical structure includes Type Ⅳ and Ⅶ collagen, laminins and heparan sulphate proteoglycans. Type Ⅳ collagen in particular is of great importance and provides the basic scaffold that supports other BM components, such as laminin networks and perlecan oligomers[52-54].

Any alteration in ECM structural profile or degradation of any of its molecules may cause cellular and tissue dysfunction and therefore disease. CRC induces numerous ECM changes, the BM included. Type Ⅶ collagen is altered or disappears during the development of multiple cancers, such as melanoma, breast and prostate. Moreover, laminin-5 is usually absent from the ECM structure in advanced CRC. Interestingly, new synthesis or accumulation of BM molecules (*e.g.*, laminin-1 or 5) may also be imposed by tumours, and this phenomenon suggests the important role of BM in tumour invasion[53,54]. Similarly, modifications in ECM adhesion molecules also occur, while neoplastic cells express the appropriate adhesive receptors on their cellular membrane. Integrin α6β4, a molecule that normally supports cellular adhesion to the BM, is such a paradigm as, following the tumour-imposed ECM modifications, it changes its role and induces actin-mediated cell motility. In this way, integrin new distribution appears to correlate with considerable CRC aggressiveness[55]. Most importantly, neoplastic cells may also interfere in ECM metabolism through proteinase expression, of the matrix metalloproteinases (MMPs) in particular. These multi-functional degrading enzymes play a critical role in carcinogenesis but also in other stages of the invasion-metastasis cascade. CRC cells may induce the expression of MMP-2 and -9 by stromal cells, either directly or *via* a paracrine mechanism, thus modifying ECM and promoting tumour development[56-58].

EPITHELIAL-MESENCHYMAL TRANSITION OF EPITHELIAL CELLS IN THE LARGE INTESTINE

Epithelial-mesenchymal transition (EMT) is a reversible morphogenetic biological process that involves the transition from stationary polarized epithelial cells to motile, multipolar or spindle-shaped mesenchymal cells. Epithelial cells are characterised by an atypical-basal polarity, the formation of tight junctions and the expression of intercellular adhesion molecules, such as E-cadherin. On the other hand, mesenchymal cells appear to be unable to build mature intercellular contacts, invade through the ECM and express molecules including fibronectin, vimentin, N-cadherin, Twist and Snail. While EMT occurs in embryogenesis and normal early development, accumulating evidence indicates that it also plays a crucial role in tumour development and metastasis; neoplastic cells usurp EMT to obtain properties that promote their detachment from their initial site and favour their migration to distant tissues[59,60].

The process of EMT is induced, promoted and regulated by multiple effectors that also regulate EMT dur-

ing embryogenesis, including the cytokines interleukin 8 (IL-8), growth factors [epidermal growth factor (EGF), platelet-derived growth factor (PDGF), TGFβ] and ECM components. Interestingly, it has been shown that CRC cells are able to produce such molecules, including IL-1α, IL-1β and tumour necrosis factor alpha (TNF-α)[60-62]. Specifically, TGFβ plays a critical role through autocrine and/or paracrine mechanisms. This growth factor may be triggered by TNF-α, which is produced by tumour-infiltrating macrophages. The binding of TGFβ with TGFβR1 and/or TGFβR2 receptors triggers the phosphorylation of Smad2 and Smad3 dimers, which dissociate from the receptors to interact with Smad4; subsequently these dimers enter the nucleus to regulate EMT[63]. Additionally, the TGFβ-Smad pathway induces the high motility group A2 gene (*HMGA2*), which mediates EMT; HMGA2 is a nuclear factor that links TGFβ with the EMT-inducing transcription factors Twist and Snail1 and 2. TGFβ/Smad activities may also be associated with PDGF and PDGF receptor signalling, such as the phosphorylation of p68 RNA helicase to trigger nuclear translocation of β-catenin through a wingless Int (Wnt)-independent pathway[64-66].

In CRC, tumour cell EMT predominantly concerns cell adhesion mediated by E-cadherin. CRC cells present either mutations of the E-cadherin gene, proteolytic degradation of E-cadherin, insulin growth factor 1-promoted internalization of E-cadherin or disarrangement of E-cadherin and β-catenin connections[67,68]. Notably, intact cadherin-catenin complexes support the normal function of intercellular adhesion and guarantee stable "adherens junctions" and an unimpaired Wnt signalling pathway (the latter being a complex protein network controlling signal transduction)[69]. Neoplastic cells along with the E-cadherin deregulation present an accumulation of Src kinase and phospho-myosin associated with EMT[70,71], as well as high expression of guanine nucleotide exchange factor Tiam 1 that promotes metastatic potential through cellular adhesion reduction and resistance to anoikis[72]. Furthermore, CRC cells present a variable level of Ras homolog gene family, member C (RhoC), a protein that functions as a switch in signal transduction, promoting reorganisation of actin and regulating cell shape. It is noteworthy that increased expression of RhoC is associated with poor prognosis, as well as with an aberrant localization and expression of E-cadherin. The disruption of E-cadherin-mediated cell adhesion contributes to CRC cell detachment and motility. Moreover, its replacement with N-cadherin, a phenomenon named cadherin switch, further promotes cancer cell translocation and invasion of the surrounding stroma. In this way, EMT favours carcinogenesis and cellular transformation towards a metastatic phenotype[73].

ANGIOGENESIS IN THE LARGE INTESTINE

Angiogenesis is defined as the formation of new vessels from preexisting ones[74]. Hypoxia or low oxygen tension is the primary triggering factor for the onset of this process. Early in carcinogenesis, tumour growth requires angiogenesis for the provision of oxygen and nutritional factors, while later new vessels provide CRC cells with a way to the systemic circulation (intravasation) and metastasis to distant sites. In normal conditions, the angiogenic process is accurately regulated by pro- and anti-angiogenic agents. In CRC, hypoxia induces the activation of hypoxia inducible factor-1 and subsequently the expression of angiogenic factors including VEGF, basic fibroblast growth factor and PDGF by CRC cells. Notably, prostaglandin E_2 directly triggers CRC cells to express VEGF, appearing as a promising therapeutic target[75,76].

Moreover, stromal cells, such as cancer-associated fibroblasts (CAFs) and tumor-associated macrophages, mast cells, neutrophils and others, also produce proangiogenic factors inducing angiogenesis. The aforementioned factors interact with their respective receptors at the endothelial cell surface initiating signalling cascades involving p38, eNOS, as well as PI3 and ERK MAP kinases; the latter induce vasodilation, endothelial cell proliferation/migration and vessel assembly. Concurrently, angiogenesis suppressor proteins are downregulated, such as thrombospondin. The resulting vascular networks of tumours are chaotic and poorly functional due to abnormal endothelial cell structure, proliferation and apoptosis; additionally, abnormal pericytes appear to be loosely attached and do not fully cover the vessels. Consequently, neoplastic vasculature is leaky, haemorrhagic, with excessive branching, which results in oxygen depletion and extracellular acidosis[77,78].

It has been demonstrated that the microvascular density has a considerable prognostic value for tumours and may predict survival in patients with CRC[79]. To study this, a colour Doppler vascularity index (DVI) (ratio of coloured pixels/total pixels per tumour section) has been introduced, in an effort to predict distant metastasis and survival in CRC patients. It was concluded that patients who had a DVI > 15% at the primary site had worse overall survival compared to patients with DVI < 15%[80]. A similar parameter is the Doppler hepatic perfusion index (DHPI) (ratio of hepatic arterial flow/total liver flow), which appeared to be increased even in patients with occult micrometastases and is predictive of metastatic liver tumour recurrence[81]. The association between angiogenesis at the primary CRC site and in the hepatic metastases is further supported by imaging techniques, such as dynamic contrast-enhanced magnetic resonance imaging and contrast-enhanced computed tomography[82].

LYMPHANGIOGENESIS IN THE LARGE INTESTINE

The lymphatic network is a major metastatic route and lymphatic metastases constitute an important prognostic indicator in solid tumours and CRC[83]. Excessive lymphangiogenesis is associated with metastasis in CRC.

Lymphatic vessel density (LVD) has been introduced as a quantitative parameter for lymphangiogenesis. *In vitro* models demonstrated that VEGF-A and VEGF-C are the major regulators of this process and their levels correlate with LVD. In addition, PDGF-BB, FGF-2, hepatocyte growth factor (HGF), angiopoietin by binding to its receptor Tie2 and sphingosine-1-phosphate (S1P) cause lymphangiogenic effects. Neoplastic cells with high levels of sphingosine kinase 1 release S1P in the ECM, which in turn may lead to paracrine-induced angio- and lymphangiogenesis[84-86]. *In vivo* models suggested new pathways for lymphangiogenesis in CRC. The high levels of VEGF-C and VEGF-D appear to direct tumour lymphangiogenesis *via* the VEGF-C and -D pathway and their receptor VEGFR-3 (present on lymphatic endothelial cells), while VEGF-A may play a regulatory role during this process[85,87]. The activation of VEGFR-3 along with the β1 integrin subunit triggers multiple signalling pathways in the lymphatic endothelial cells, including Pyk2, NF-κB, ERK and JNK MAP kinases, which mediate proliferation and survival[88]. A recent study by Du *et al*[89] on human CRC samples indicated that metastasis associated protein 1 (MTA-1), which is expressed in various epithelial cancers and plays a critical role in metastasis, correlated with VEGF-C and mediated its expression. Hence, it was suggested that MTA-1 promotes lymphangiogenesis and therefore CRC metastasis.

Lymphangiogenesis may be initiated with the formation of lymphatic vessels by circulating endothelial progenitors (CEPs); the latter constitute a subpopulation of circulating endothelial cells derived from the bone marrow. It has been shown that CEPs are increased in the circulation in multiple pathologies, cancer included[27,90,91]. However, alternative pathways for the initiation and progress of lymphangiogenesis may also exist. The differentiation of other cells into lymphatic endothelial cells, such as macrophages and stromal cells, may be such pathways[92,93].

Interestingly, apart from invasion to lymph nodes, CRC LVD is also associated with liver metastasis. Although this association is rather unclear, a possible explanation may be that CRC cells express chemokine receptor CXCR5; its ligand BCA-1/CXCL13 is mutually expressed on lymphatic endothelial cells and the liver[94]. CRC cells may be directed through the lymphatic network to both lymph nodes and the liver. Moreover, lymphatic metastases are traced at the liver lymphatic drainage network, including portal, mediastinal and coeliac lymph nodes. These liver-associated lymphatic metastases may be generated from previous CRC liver metastases. All these findings show clearly the importance of angio- and lymphangiogenesis for carcinogenesis and metastasis and explain why they attract great research interest and constitute promising targets for anticancer therapy[27,95].

INTRAVASATION AND CIRCULATION - SURVIVAL IN THE LYMPHATIC AND/OR THE BLOOD VESSELS

Malignant cell detachment from their primary tumour is the necessary step for their intravasation into existing or newly formed lymphatic or blood vessels. Certain molecular changes concerning the expression of degrading enzymes (such as the MMPs) and adhesion molecules (such as selectins, integrins and others) appear to contribute to CRC cell intravasation. Notably, the tortuous leaky neoplastic blood network, with its loose junctions among endothelial cells and the defective pericyte coverage, may substantially promote tumour cells entering into the lumina of vessels[78].

Sonoshita *et al*[96] studied murine and human tissue and showed that the transcriptional modulator enhancer of split (Aes) prevented CRC cells from intravasation through the impairment of trans-endothelial invasion that followed Notch-dependent mechanisms. Concurrently, urokinase plasminogen activator (uPA) expression is increased in patients with FAP during the transformation of normal to dysplastic epithelia[97,98]. The uPA system consists of uPA, the uPA receptor (uPAR), the tissue-type plasminogen activator (tPA), the plasminogen (Plg) and plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). This biological system is involved in multiple molecular pathways, including adhesion, chemotaxis, cytokine release, protease expression, neutrophil proliferation and activation for oxidant production. It modulates inflammation, growth, invasion, angiogenesis and metastasis of multiple tumour types[99,100]. When uPA connects with uPAR, it activates Plg along with other proteases such as MMP-2 and -9. These events promote ECM fragmentation and CRC cell detachment and intravasation.

Interestingly, when normal cells lose their contact with the ECM and their neighbouring cells, they undergo apoptosis that is triggered by broken integrin bonds. The ability of CRC cells to survive in the presence of fragmented ECM constitutes a crucial property of metastatic cells. This apoptosis resistance might be attributed to the overexpression of focal adhesion kinase by CRC cells, which contributes to conferring survival by activating certain molecular pathways, including ERK and AKT. Hence, tumour cells can by-pass integrin signalling and survive without contact with the ECM[101,102].

Following their detachment, access to the blood and lymphatic vessels is the next step for metastatic CRC cells. The microvessel diameter and their leaky structure are important factors that promote passive CRC cell entry into the circulation. Although lymphangiogenesis is less studied than angiogenesis, it appears that LECs, stimulated by VEGF-C, secrete mitogenic and/or chemotactic factors, which may attract tumour cells to adhere to, pass through and intravasate into the newly-built lymph vessels[90,103].

CRC cells are transported through the hepatic-portal circulatory system to reach the liver. The inferior and superior mesenteric veins and the portal vein, along with their neighbouring lymphatic vessels, constitute the dominant metastatic routes for hepatic metastases. In cancer patients, a large number of circulating tumour cells may be detected, although the intravasated cellular populations do not correlate with the density and the extension of metastases. Accumulating experimental evidence has

suggested that entrance into the systemic circulation is lethal for the vast majority of tumour cells; only 0.01% of metastatic cancer cells may trigger the formation of metastasis when injected into the systemic circulation[27]. *In vitro* studies using videomicroscopy to monitor intravasated cancer cells have indicated that most of the cells undergo apoptosis during their passage through the vascular wall or soon after their entrance into the circulation. It is generally accepted that two major mechanisms contribute to tumour cell death following their intravasation: mechanical stress and the immune system[5,104].

Haematogenous dissemination exposes tumour cells to the strong mechanical forces generated by blood flow. In particular, when they circulate within a narrow capillary network or the microvasculature of contractile organs, such as the heart and the skeletal muscles, they are forced to transform their shape from spherical to cylindrical, which is lethal to the majority of tumour cells[5]. However, as tumour cells get progressively more invasive and metastatic through a series of mutations, they display larger cell deformations and shape alterations. *In vivo* studies have demonstrated that metastatic cells are quite deformable and both the cell nucleus and cytoplasm may undergo strong compression and shape deformation in small capillaries. Specifically, the length of the cell nucleus may increase 1.6-fold and the cytoplasmic major axis up to 3.9-fold in comparison with the same cells in larger microvessels[105-107]. Also, tumour cells reaching capillary bifurcation points can stretch and extend their cytoplasms in both directions[105,106]. Mechanical forces, such as endothelial contraction, shear or pulsatile stresses, cause the production of oxygen radicals including NO and ROS, which generate apoptosis of CRC cells.

Metastatic tumour cells have developed multiple molecular pathways in order to survive the mechanical forces in the circulation. CRC cells interact with platelets and/or themselves to form large emboli that protect them from shear stress[108]. Cancer cells may activate adhesion pathways involving integrins, leading to their attachment to endothelial cells and thus evasion of anoikis. Programmed cell death may also implicate metabolic pathways including pentose phosphate and control of glucose uptake[109]. Moreover, the tyrosine kinase TrkB was demonstrated to suppress anoikis and appeared to be crucial for metastatic progression in CRC cells[110].

The second major mechanism responsible for metastatic cell elimination in the circulation is immunosurveillance. Numerous cytokines released by cancer cells, endothelial cells or immune cells, such as IL-2, -12 and -18, may activate various subsets of immune cells including T-lymphocytes and natural killer (NK) cells. The latter eliminate cancer cells through Trail and/or NKG2/perforin pathways. Similar to mechanical forces adaptations, metastatic cells have developed mechanisms to evade immunosurveillance. It has been shown that cancer patients present high levels of acute phase glycoproteins in their blood, correlated with the disease. These proteins may protect malignant cells against anoikis and immune cell attacks[111]. Furthermore, tumour cells interact with platelets forming large emboli, a process mediated by the expression of tissue factor and L- and P-selectins. In this way, they shield themselves against both immune detection and shear forces[112]. Undoubtedly, immune system and cancer cell interactions attract great research interest and the manipulation of immunesurveillance is a major strategy in anticancer treatment.

EXTRAVASATION - CRC CELL ARREST IN THE HEPATIC SINUSOIDS

The sinusoids constitute the specific hepatic capillary network where four different cell populations reside within the sinusoidal lumen or in proximity to the sinusoidal wall: sinusoidal endothelial cells (SECs), Kupffer cells (KCs), hepatic stellate cells (HSCs) and pit cells. Each of these cell types plays a crucial role in hepatic homeostasis and CRC metastasis[113].

The progression of CRC hepatic metastasis is divided into four interrelated phases: (1) microvascular phase of liver-infiltrating malignant cells; (2) interlobular micrometastasis phase; (3) angiogenic micrometastasis phase; and (4) established hepatic metastasis phase. The first phase mainly occurs within the sinusoids, whereas the following ones describe further metastatic steps that affect the inner hepatic parenchyma. Although sinusoidal cells contribute to all four, they predominantly mediate the first phase[114].

During the microvascular phase, resident cells generate multiple tumouricidal and tumourigenic effects, which may either promote the invading cell elimination or liver colonisation. A plethora of molecular pathways are involved, including NO and reactive oxygen release by SECs, expression of adhesion molecules, such as selectins, integrins and others by the same cells, phagocytosis, cytokine and growth factor release by KCs and HSCs, release of cytotoxic agents from pit cells. The aforementioned examples demonstrate the complexity and the importance of the intercellular reactions that occur within the sinusoids in colorectal liver metastasis[114-116].

Sinusoidal endothelial cells

SECs were first described by Wisse at the beginning of 1970s. Their cytoplasm includes characteristic canals, arranged in sieve plates named fenestrae. Fenestration constitutes a unique marker and distinguishes SECs from all other endothelial and liver cells (Figure 4)[117,118]. They form a major scavenger cell system and accomplish receptor-mediated endocytosis and pinocytosis through three major types of endocytic receptors: the liver sinusoidal cell mannose receptor, the liver sinusoidal cell scavenger receptor and the liver sinusoidal cell receptor IIb2, which is an Fc-γ receptor[114]. The scavenging properties of SECs are critical in CRC metastasis. *In vivo* murine experiments indicated that autotaxin, a phosphodiesterase that promotes metastasis and angiogenesis, was rapidly removed from the blood circulation and degraded by SECs[119].

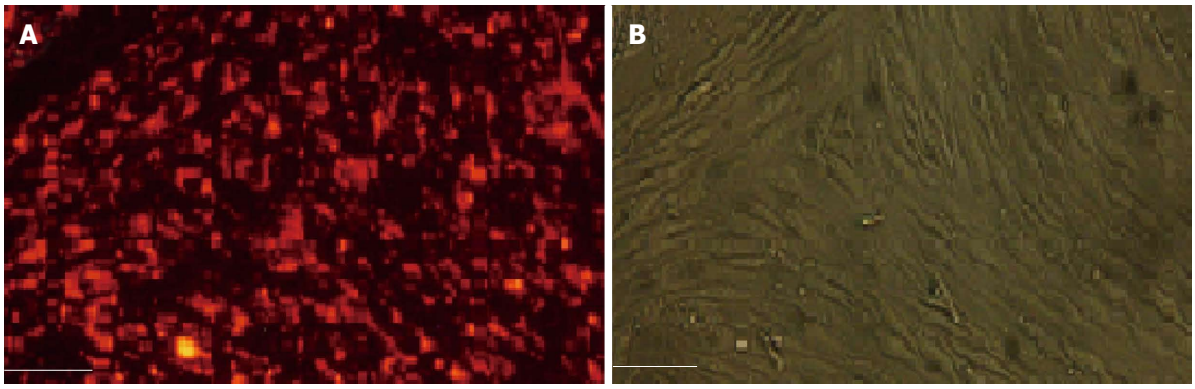

Figure 4 Sinusoidal endothelial cells. Fluorescence labeled (AcLDL) (A) and light microscopy (B). The cells have a cobblestone architecture on the sixth day in culture (authors' archive). Magnification × 200. Scale bars = 100 μm. AcLDL: Acetylated low density lipoprotein.

Table 1 Molecules produced by Kupffer cells that may mediate their interactions with metastasising colorectal cancer cells within the hepatic sinusoids[126,127]

Group	Molecules
Cytokines and chemokines	Tumour necrosis factor alpha
	Transforming growth factor beta
	Interleukins (IL-1α, -1β, -6, -8, -10, -12 and -18)
	Interferon gamma
	Platelet-activating factor
	Monocyte chemotactic protein-1
	Macrophage inflammatory protein (MIP-1)
Hydrolytic and proteolytic enzymes	Urokinase-type plasminogen activator
	Metalloproteinases (MMP-1, -7 and -13)
Lipid metabolites (prostanoids)	Prostaglandin E2
	Thromboxane
Oxygen species (superoxide) and hydrogen peroxide	
Nitrogen species (nitric oxide)	

This study further supported previous data which demonstrated that hepatectomy or chemically-induced injury resulted in an increase of serum autotoxin[120].

Although SECs appear to play a tumouricidal role, they may also aid tumour cells to arrest and metastasise within the liver. Under cytokine activation, SECs may express adhesion molecules, such as E-selectin, which mediate cancer cell attachment to the endothelium, thus facilitating their extravasation into the hepatic parenchyma[121,122].

Experimental studies have attempted to investigate and exploit SEC properties in anticancer therapy. Gene therapy uses certain vectors, such as adenoviruses, to reach target cells and is considered a new and promising treatment of acquired diseases, including liver neoplasms and metastases. SECs, in conjunction with KCs, may endocytose and destroy these vectors, cancelling any therapy. On the other hand, as hepatocytes constitute the primary target cells for anti-cancer therapy, gene vectors should reach the space of Disse, in order to interrelate with hepatocytes. The space of Disse may be accessible only through SEC fenestration, and the diameter of the latter ranges according to species and liver condition; ageing or liver diseases substantially alter fenestrae, impairing the vector access to the space of Disse[123].

Kupffer cells

These constitute the biggest (more than 80%) tissue macrophage population in the body of vertebrates and approximately 15% of all liver cells[124,125]. KCs mainly act as scavengers around the sinusoids, but also as antigen-presenting cells and liver regeneration mediators. When activated, KCs produce a wide variety of molecules, such as growth control mediators, inflammatory agents, proteolytic and hydrolytic enzymes, oxygen and nitrogen species and lipid metabolites. All these products modulate acute and chronic liver responses to pathogens, chemicals, drugs, injury, as well as cancer and metastasis (Table 1)[126-128].

KCs play a crucial role in the "host tumoural surveillance system". As they constantly reside around the sinusoids, they discriminate and remove neoplastic cells that reach the liver. Interestingly, CRC cells become vulnerable to macrophage tumouricidal activity during endothelial adhesion and extravasation[129,130]. Specifically, KCs may recruit inflammatory cells, they may arrest CRC cells and inhibit their growth acting in a cytostatic way, and they may bind and eliminate them in a cytotoxic way[130]; the latter occurs by several mechanisms: phagocytic release of TNF, secretion of proteases and production of oxygen metabolites[131-133].

Rat experiments demonstrated that in the early stages of CRC liver metastasis, KCs exerted tumouricidal activity in conjunction with NK cells. One hour after CRC cells had reached the liver, more than 70% were already in contact with KCs; the KC population was considerably increased from the first day and phagocytosed more than 90% of malignant cells. However, NK depletion left 35% of the cancer free from KC interactions. The responsible mechanism may be that activated NK cells secrete pro-inflammatory cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF) and interferon gamma (IFN-γ), which in turn activate KCs or sensitise tumour cells to cytotoxic effects. Alternatively, NKs may induce CRC cell apoptosis, causing exposure of phosphatidylserine and enhancing phagocytosis by KCs[115]. The opposite interaction was also reported: activated KCs may produce IL-12 and/or IL-18, which enhance IFN-γ release by pit cells that exhibit high tumouricidal activity,

thus inhibiting CRC haematogenous metastasis in murine livers[134,135]. It appears that in the metastatic process, KCs and pit cells act in close cooperation against the invading cancer cells. Both produce cytokines and interact stimulating one another, eliminating cancer cells directly or mediating cancer cell death by their counterparts.

High mobility group box 1 (HMGB1) is a protein produced by normal and cancer cells which triggers apoptosis in macrophages and monocytes. Experimental data from Dukes' C and D surgical specimens showed that HGMB1 levels in the portal blood were higher for Dukes' D and correlated with the levels in the primary tumours. When HMGB1 concentration was raised, KC numbers were substantially decreased. Moreover, *in vitro* murine experiments performed in the same study showed that the administration of the protein decreased KC populations and promoted liver metastases[136]. These findings support the tumouricidal role of KCs in the metastatic process.

The interaction between KCs and invading tumour cells is not always in favour of liver homeostasis. Binding to KCs facilitates CRC cell arrest in the liver; if the killing process is not accomplished promptly or is partially completed, then the binding process substantially contributes to liver colonisation. Experimental data in rats advocated that KCs exert a limited capacity for tumour surveillance; when malignant cells reach the liver in very high numbers or present great antigenic diversity, KCs are eventually saturated and metastasis progresses[130].

Additionally, KCs produce growth factors, such as HGF, which facilitate tumour cell proliferation[137]. Furthermore, the ability of KCs to secrete MMPs, especially MMP-9 and MMP-14, as well as their inhibitors, may enhance angiogenesis and tumour invasion, *via* alterations of the ECM[138]. Notably, MMP-9-deficient mice presented considerably fewer liver metastatic lesions when CRC cells were injected in their spleen. MMP-9 was primarily derived from KCs, independently of its expression by tumour cells[139]. Moreover, *in vitro* experiments have indicated that highly malignant cells reduce, in their favour, the phagocytic capacity of KCs and promote colonisation[140]. The preceding experimental evidence suggests that KCs prevent tumour outgrowth and liver metastasis when the burden and the rate of invading cells are relatively low. However, KCs may contribute to liver colonisation if their tumouricidal ability is saturated due to excessive numbers of invading cells or when metastases are already established.

KCs present an 80 kDa carcinoembryonic antigen (CEA) receptor, which mediates binding and subsequent degradation of CEA[141,142]. When CEA is complexed, KCs are activated and secrete through β-2 adrenergic pathways large amounts of cytokines, including TNF-α, IL-1β, IL-6 and IL-10[141,143]. These pro- and anti-inflammatory agents generate alterations in the sinusoidal endothelium and SECs express adhesion molecules of the selectin family, which promote the arrest and extravasation of tumour cells[144]. Murine studies indicated successful adhesion of CRC cells to KCs and SECs, without the immediate mediation of CEA, excluding CEA's function as an adhesion molecule in hepatic colonisation[145]. The last observation was further investigated and it was revealed that CEA supports CRC cell survival *via* the induction of IL-10 and subsequent decrease of NO concentration. IL-10 is probably released by stimulated KCs and NO levels decrease due to inhibition of inducible nitric oxide synthetase[146].

Accumulating data have demonstrated the important role of immunoglobulin superfamily adhesion molecules in colorectal liver metastases, referring to KCs. Murine experiments demonstrated that CRC cells trigger KCs to produce pro-inflammatory cytokines, which in turn stimulate SECs to express high levels of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)[147] or VCAM-1 and platelet endothelial cell adhesion molecule 1[148]. These molecules mediate cancer cell adhesion, follow E-selectin expression and promote subsequent extravasation. Similarly, *in vitro* experiments using CEA-activated murine KCs indicated that cytokines were released, which activated endothelial cells to express ICAM-1 and VCAM-1[122,149].

Consequently, several lines of evidence support the hypothesis that when CRC cells reach the hepatic sinusoids, they secrete CEA that may activate KCs to produce cytokines; the latter stimulate SECs to express cell adhesion molecules that bind colorectal metastasising cells. This mechanism obviously contributes to malignant cell arrest, proliferation and invasion (Figure 5).

The cytostatic and cytotoxic actions of KCs were taken into consideration in liver metastasis research and adjuvant strategies were created in order to maintain and increase their potential antitumour role. As transient peri- and post-operative immunosuppression has been observed in patients undergoing major surgical interventions, further enhancement of KC activities is particularly useful during and after hepatectomy[150]. GM-CSF was tested for this purpose and experimental data from human liver wedge biopsies showed that it could stimulate KCs to exert increased cytotoxicity against a SW948 colon carcinoma cell line[151]. In syngenic rat models, the administration of this factor increased KC numbers and significantly enhanced their cytotoxicity *ex vivo*, cancelling the development of small metastatic foci[152]. IFN-γ was also tested targeting KC activity; in syngenic murine models, the preoperative administration of IFN-γ caused high anti-metastatic function of KCs and NKs in early liver metastasis[153]. These promising results remain to be further analysed and repeated in bigger studies using human hepatic tissue.

Hepatic stellate cells

HSCs are located in the space of Disse, comprising about 15% of the nonparenchymal hepatic cells. They have a unique morphology, due to long cytoplasmic processes that form a spindle-shaped cellular body (Figure 6)[154]. KCs produce chemokines that induce mono- and poly-

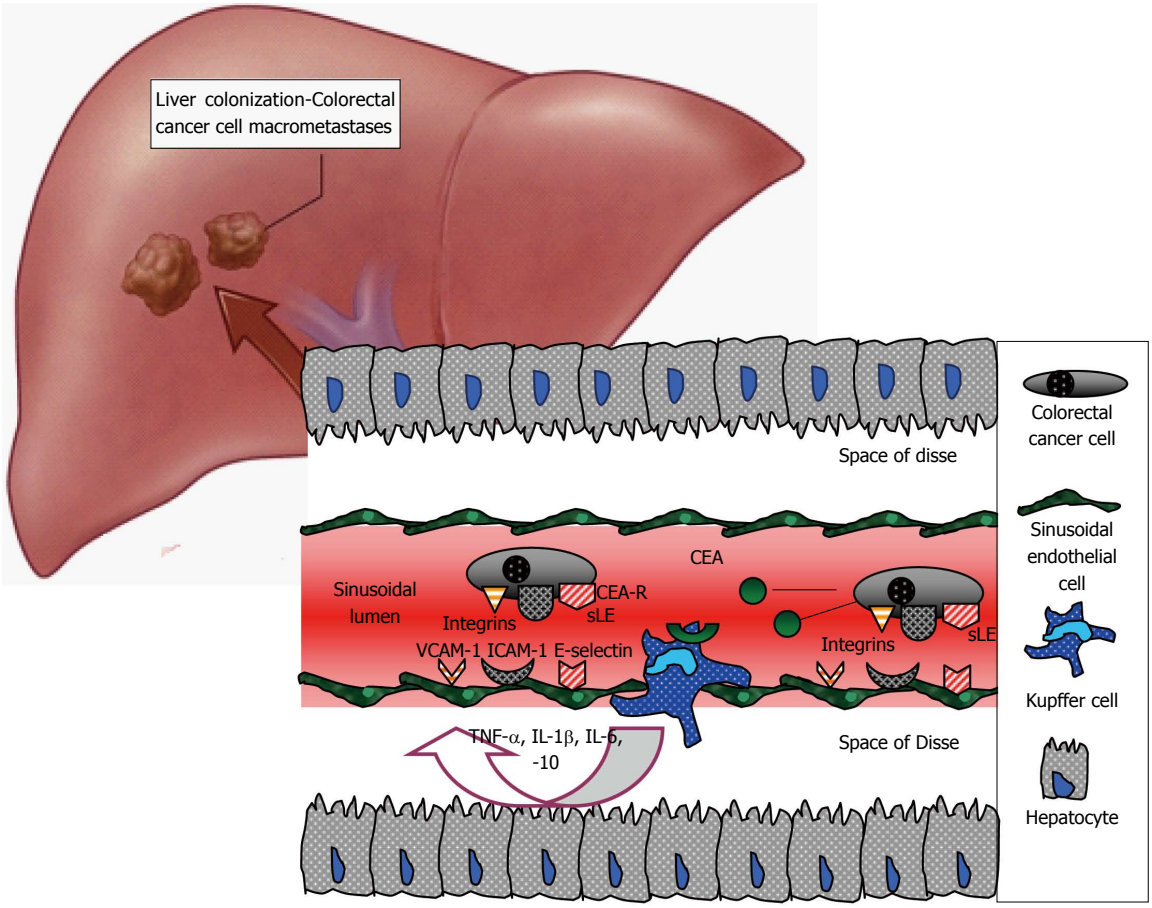

Figure 5 The role of Kupffer cells in colorectal cell adhesion-arrest within the sinusoids during haematogenous liver metastases. Kupffer cells are activated through carcinoembryonic antigen (CEA), release multiple chemokines and stimulate sinusoidal endothelial cells to express adhesion molecules, inducing colorectal cancer cell arrest. CEA-R: CEA receptor; ICAM-1: Intercellular adhesion molecule 1; IL: Interleukin; sLE: Sialyl Lewis antigen; TNF-α: Tumour necrosis factor alpha; VCAM-1: Vascular cell adhesion molecule 1.

morphonuclear leukocyte infiltration, activate neutrophils and control lymphocyte populations exerting immunoregulatory activity[155,156]. Furthermore, HSCs act as antigen-presenting cells that may activate T lymphocytes[157,158] and release pro-inflammatory cytokines when exposed to bacterial endotoxins through toll-like receptors[158,159].

HSCs secrete and respond to a wide variety of cytokines. They modify the activity of various growth factors, express adhesion molecules, such as ICAM-1 and VCAM-1, and regulate the detoxification of ethanol and xenobiotics[156,160]. Activated HSCs display contractility, chemotaxis, fibrogenesis, matrix degradation activity, proliferation, and retinoid loss. They mediate inflammation, cell survival and apoptosis, liver regeneration and monitoring of cellular pH[161-163].

HSCs are also key cells in tumour growth and CRC metastatic processes. Experimental studies in rats revealed that conditioned media from cultures of hepatocellular carcinoma hepatocytes could activate HSCs[164]. Moreover, *in vitro* experiments with metastatic melanoma cells concluded that tumour cells triggered HSC activation; the latter promoted angiogenesis through VEGF expression[165]. Injection of colon carcinoma cells in nude mice favoured the formation of hepatic metastatic foci through HGF and TGF-β1 produced by HSCs. Similarly, tumour cells secreted PDGF-AB and enhanced HSC proliferation and locomotion[166].

Co-cultures of SECs and HSCs caused spontaneous cellular differentiation, with HSCs forming the core and SECs the surface of the cell population. Concurrently, activated HSCs cultured with SECs expressed functional smooth muscle cell phenotype and formed capillary-like structures in angiogenesis assays. Taking into consideration that tumours activate HSCs, their contribution to neoangiogenesis through interactions with SECs was implicated in these studies[114,167].

Pit cells

The name pit cell is related to their cytoplasmic granules. The cells contain granules with lysosomal enzymes, perforin and various phosphatases, but their structural characteristic is the presence of cytoplasmic rod vesicles. Their shape varies, due to the presence of pseudopodia[113,168,169].

Pit cells substantially contribute to hepatic immunity and exert strong antitumour action. In collaboration with KCs, they represent the first line of liver defence against cancer cells. Experimental studies in rodents demonstrated that pit cells are highly cytotoxic against multiple

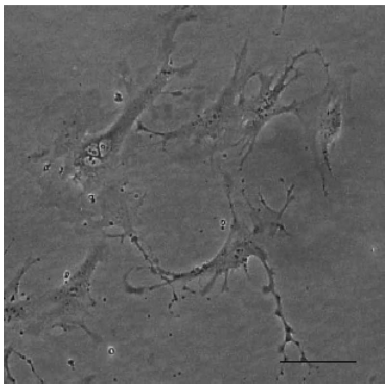

Figure 6 Hepatic stellate cells with their cytoplasmic processes, after 4 d in culture (authors' archive). Light microscopy (magnification × 200). Scale bar = 100 μm.

malignant cell lines, including murine fibrosarcoma L929, colon carcinoma CC531 and colorectal carcinoma DHD-K12 rat cells[169].

Pit cells exert their cytotoxicity through binding to target cells, a process named conjugation. Various adhesion molecules on pit cells mediate this process, such as CD2, a member of the immunoglobulin superfamily, CD28 and lymphocyte function-associated antigen 1, while CD54 and CD58 may be present on target cells[170,171]. In addition, interactions between β_2 integrins and ICAMs are crucial, supporting these cell-cell conjugations[169,170,172].

Following conjugation, various receptors may be stimulated, triggering or inhibiting pit cell cytotoxicity. Three superfamilies of NK cell receptors are presented primarily on human pit cells, while others, named co-receptors, still remain under investigation: the killer immunoglobulin receptor that recognizes major histocompatibility complex (MHC) class I molecules, the c-type lectin, recognising non classical MHC class I or class I-like molecules, and the natural cytotoxicity receptor superfamily, which remains to be further studied[173].

Pit cell tumouricidal actions are exerted mainly through three mechanisms[168,174], as follows:

Perforin-granzyme pathway: Through calcium-dependent molecular reactions pit cells adhere to tumour cells and release perforin and proteases into the intercellular space. Subsequently, perforin induces pores in the tumour cytoplasmic membrane and then proteases may generate DNA segmentation.

Apoptosis pathway: Pit cells express both the FasL and the tumour necrosis factor-related apoptosis-inducing ligand. When pit cells interact with tumour cells, these ligands bind to respective receptors and cancer cells undergo apoptosis.

Cytokine pathway: Pit cells secrete cytokines, such as IFN-γ, and thus activate lymphocytes and macrophages against invading cancer cells.

LIVER COLONISATION

The adherence of metastasising CRC cells to SECs is a primary step of great importance toward liver invasion and colonisation[140]. Malignant cells bind to SECs initially through selectins. However, these bonds do not appear to be strong enough to guarantee stable cell adhesion. Integrins are necessary to stabilise tumour cell-SEC adhesion. If integrins do not conform, then the bonds are broken and cancer cells are released into the blood or undergo mechanical damage[175]. The development of strong intercellular bonds allows CRC cells to resist the attractive forces of plasma flow and circulating blood cells, when they adhere to the hepatic sinusoids[176]. Multiple signalling molecules, including focal adhesion kinase, paxillin, and cytoskeletal proteins, are probably required for tumour cell adhesion and stabilisation under the hydrodynamic conditions of blood flow[175].

Shimizu *et al*[177], studying CRC liver metastases in murine models, reported that shortly after endothelial adhesion occurred within the sinusoids, metastasising cells extended cytoplasmic projections towards the space of Disse, through the pores of SECs. Forty-eight hours after their injection in mice, CRC cells may reach the hepatocytes and enter their cytoplasm, and 72 h later, they developed metastatic foci.

From the moment of extravasation, cytotoxic T cells, monocytes and macrophages which occupy extra-sinusoidal hepatic tissue are activated against the metastatic cells, though not always successfully[178]. Ultimately, few CRC cells cause micrometastases in the hepatic parenchyma. They remain in a dormant state, the duration of which is unknown. It is probable that these micrometastases will be reactivated after an unspecified time period and will create macrometastases. The last stage of the invasion-metastasis cascade is then accomplished[179,180].

It has been proposed that carcinoma cells address the problem of an incompatible microenvironment at the distant metastatic site through the establishment of a "premetastatic niche"[181]. In that state, CRC cells may release soluble CD44 that mediates resistance to apoptosis[182]. The crosstalk among cancer cells, immune cells, endothelial and stromal cells causes the production of various chemokines and growth factors, such as the EGF and TGF-α, which promote metastatic cell growth in the liver. Interestingly, in order to survive and develop a secondary neoplasm in the liver, CRC cells must undergo the reverse transition from EMT, which is termed mesenchymal-epithelial transition (MET). Consequently, CRC cells express epithelial cell markers such as E-cadherin[183,184]. Additionally, Belluco *et al*[185] showed that CRC cell kinase profile at the hepatic metastatic sites differs considerably from the initial intestinal site. These data indicate that CRC cells adjust their signalling network at the hepatic microenvironment in order to survive and generate metastatic foci. The activation of MET is probably triggered by the liver ECM. Hepatic CAFs appear to promote this process through overexpression of COX2 and TGF-β2.

Table 2 Adhesion molecules with potential prognostic value in colorectal cancer progression and hepatic metastases[176,194]

Adhesion molecule family	Adhesion molecule	Expression
Cadherins	E-cadherin	Colon epithelial cells
	P-cadherin	Colon epithelial cells
Lectins	sLEx	Colon epithelial cells
	sLEa (CA 19-9)	
	Galectin-3	
Selectins	E-Selectin	ECs
	L-Selectin	Leukocytes
	P-Selectin	ECs and platelets
Integrins	LFA-1 ($\alpha_L\beta_2$)	Colon epithelial cells, ECs, fibroblasts, leukocytes and platelets
	VLA-4 ($\alpha_4\beta_1$)	
Immunoglobulin superfamily CAMs	ICAMs	ECs, fibroblasts and leukocytes
	PECAM-1	ECs, platelets and leukocytes
	VCAM-1	ECs and epithelial cells
	MadCAM	Colon epithelial cells
	CEA	Colon epithelial cells
Proteoglycan receptors	CD44	Colon cells and ECs

CA 19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; EC: Endothelial cell; ICAM: Intercellular adhesion molecule; LFA-1: Leukocyte function-associated antigen 1; MadCAM: Mucosal addressin cell adhesion molecule; PECAM: Platelet endothelial cell adhesion molecule; sLEx: Sialyl Lewis x antigen; VCAM: Vascular cell adhesion molecule; VLA-4: Very late activation antigen 4.

The transition of metastatic microcolonies to macroscopic metastases may occur after weeks or months or they may persist as microcolonies in a state of long-term dormancy. However, similarly to the initial CRC tumours, this transition also needs additional vascular supply through neoangiogenesis. VEGF-A is the major growth factor that regulates neovascularisation and VEGFR1 is the main receptor.

CONCLUSION

The invasion-metastatic process from the initial site at the large intestine to the liver is a long process, where multiple molecular pathways and numerous cell types are involved. Recent research has elucidated various aspects of this process, such as the role of EMT and stromal microenvironment cellular crosstalk, the importance of adhesion molecules (Table 2), the significance of proteases, as well as the role of VEGF members in angio- and lymphangiogenesis.

Furthermore, technological advances have revolutionised the study of metastasis, since imaging techniques have provided real-time visualisation of metastatic cells in the ECM and the circulation[186]. Also, new genetic, molecular and biochemical techniques may permit the investigation of tumour cell heterogeneity at the initial and their metastatic sites and may explain its functional significance[187].

The progress in basic research concerning CRC hepatic metastasis over the last decade has been accompanied by the rapid translation of experimental data to oncological treatment. An anti-VEGF monoclonal antibody, named bevacizumab, has been introduced in CRC therapy and has contributed to prolonged patient survival[188]. Concurrently, clinical trials are in progress for antiCEA antibodies[189], MMP inhibitors[190], antibodies against integrins[191] or molecules that increase immunosurveillance[192,193]. Although comprehension of CRC hepatic metastasis has substantially evolved, the invasion-metastasis cascade remains partially understood and future basic and clinical research still has multiple issues to clarify.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Colorectal cancer and immunity: What we know and perspectives

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Abstract

Strong evidence supports the concept of immunosurveillance and immunoediting in colorectal cancer. In particular, the density of T CD8$^+$ and CD45$^+$ lymphocyte infiltration was recently shown to have a better prognostic value than the classic tumor node metastasis classification factor. Other immune subsets, as macrophages, natural killer cells or unconventionnal lymphocytes, seem to play an important role. Induction of regulatory T cells (Tregs) or immunosuppressive molecules such as PD-1 or CTLA-4 and downregulation of antigen-presenting molecules are major escape mechanisms to antitumor immune response. The development of these mechanisms is a major obstacle to the establishment of an effective immune response, but also to the use of immunotherapy. Although immunotherapy is not yet routinely used in colorectal cancer, we now know that most treatments used (chemotherapy and biotherapy) have immunomodulatory effects, such as induction of immunogenic cell death by chemotherapy, inhibition of immunosuppression by antiangiogenic agents, and antibody-dependent cytotoxicity induced by cetuximab. Finally, many immunotherapy strategies are being developed and tested in phase Ⅰ to Ⅲ clinical trials. The most promising strategies are boosting the immune system with cytokines, inhibition of immunoregulatory checkpoints, vaccination with vectorized antigens, and adoptive cell therapy. Comprehension of antitumor immune response and combination of the different approaches of immunotherapy may allow the use of effective immunotherapy for treatment of colorectal cancer in the near future.

Key words: Colorectal cancer; Immunotherapy; Immunity; Immunoregulation; Vaccination

Core tip: Immune system is now widely accepted as a key mechanism to prevent occurrence of cancer and intratumoral T CD8$^+$ and CD45$^+$ lymphocytes infiltrate has shown to be a major prognosis factor in colorectal cancer. However, immunity fail in controlling tumor growth, because of strong escape mechanisms to the immune system developed by the tumor. In recent years, several immunotherapy strategies have been tested in colorectal cancer. This review provides an understanding of the mechanisms involved and identifies innovating therapeutic strategies.

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INTRODUCTION

With around 1 million new cases every year, colorectal cancer (CRC) is the third most frequent cancer in the world. Despite recent therapeutic advances it causes more than 500000 deaths every year. So there is a real need for therapeutic progress to reduce the risk of recurrence after surgery or prolong survival of patients with metastatic disease. Advances could be provided by understanding the role and mechanisms of the immune response in CRC and by the development of immunotherapy. Indeed there is growing evidence that the immune system may play a role in preventing the occurrence, growth and metastatic diffusion of tumors.

The aim of this review is to provide a comprehensive analysis of known mechanisms of immune response against CRC and immune escape strategies developed by tumor cells, and to present current and future perspectives in immunotherapy for CRC. In particular we will focus on the following questions: (1) What is the clinical and prognostic impact of natural immune response mechanisms? (2) What are the escape mechanisms developed by the tumor which limit the efficiency of the immune system and/or immunotherapy? (3) What is the impact of the immune system in the therapeutic effect of current standard treatments? or (4) Can we in the future develop effective immunotherapy for CRC management?

BASIC CONCEPTS IN ANTITUMOR IMMUNITY

Immune surveillance

The role of immunity in cancer was suspected in 1909 by Ehrlich, who speculated that the immune system can repress the growth of carcinomas. About 50 years later, Macfarlane Burnet and Lewis Thomas elaborated the concept of immunosurveillance, as the capacity of the immune system to promote an effective immunologic reaction to tumor cell-specific neoantigens that eliminates developing cancer before clinical expression.

However, this concept of immunosurveillance has long been questioned. When Hanahan established the six criteria necessary for the development of a tumor in 2000, immunity was not cited.

In humans, the role of immune surveillance was first suspected with observation of increased occurrence of cancer in patients with immunodeficiency. Cohorts of transplanted patients and HIV-infected subjects in particular showed a strong increase in the incidence of cancers[1,2]. But in humans as in murine models the increase in occurrence of neoplasias has long been explained as a consequence of carcinogenesis related to certain infectious pathogens (EBV, HPV, HIV...). However, melanoma, renal, lung, pancreatic and colon cancer are non-pathogen-related and an increased incidence of these tumors was reported in immunocompromised patients. Registries and meta-analyses of solid organ transplant recipients have shown an increased risk of CRC[3,4] with a standardized incidence ratio of 1.2 to 1.8, this increased risk is more controversial in HIV-infected patients[5].

The anti-tumor immunosurveillance concept was finally demonstrated in animal models by Shankaran *et al*[6], who observed the occurrence of spontaneous neoplasias in immunocompetent or immunodeficient mice. Mice were kept under aseptic conditions for 15 to 21 mo. During this observation period immunocompetent mice did not develop any malignant tumors, while RAG2-/- mice deficient in T and B lymphocytes developed malignant colon and lung tumors (not known to be associated with an infectious agent) in about 50% of cases, and RAG2-/- STAT1-/- mice deficient in T and B lymphocytes and insensitive to IFNγ developed neoplasia in 80% of cases. Since then, many studies have shown the involvement, depending on the model, of the innate and/or adaptive immune response in the protection against the occurrence of malignant neoplasms.

Immunoediting and immune escape

The immunosurveillance concept was then completed by that of immunoediting[7], which describes the interactions between the immune system and the tumor, allowing cancer cells to escape immune surveillance. The selection pressure exerted by the immune system on tumor cells allows the emergence of resistant clones. According to the theory of immunoediting, immune escape occurs in three phases: the immunosurveillance period with the elimination of tumor cells by the immune system, the latency period, corresponding to a state of equilibrium, and the phase of escape, allowing tumor progression and clinical expression.

ANTITUMOR IMMUNITY IN COLORECTAL CANCER

Innate immunity

Natural killer (NK) cells play a crucial role in preventing recurrence, and are a prognostic factor: NK cells play a major role in the immune response to cancer. They help to prevent tumors, and control tumor growth and dissemination, as shown in murine[8,9] and human models[10,11]. NK cells have 2 types of receptors: activating receptors, including NKG2D, and killer inhibitory receptors (KIR). The NKG2D receptor can bind different activating ligands overexpressed on cancer cells. On the other hand, KIR recognize major histocompatibility (MHC) class I molecules and NK cells can thus also be activated by the decreased expression of MHC class I molecules reported on cancer cells. These two mechanisms can activate NK cells against tumor cells. In

addition, NK cells may exert a cytotoxic effect against cancer cells through other mechanisms such as antibody-dependent cell-mediated cytotoxicity (ADCC), and secretion of cytokines including IFNγ[12].

In CRC, an extensive intratumoral infiltration of NK cells has been reported to be associated with a better prognosis[13]. Moreover, a direct correlation between increased outcome and NK cell infiltrates is suggested[14]. In particular, NK cells could be involved in protection against cancer-initiating cells (CICs)[15]. CICs are characterized by slow growth and resistance to drugs and radiation, and play a crucial role in tumor recurrence. Recent data suggest that CICs are more sensitive to NK cells because they strongly express activating ligands as NKP30 and NKP44 and express low levels of MHC class I molecules.

Unconventional lymphocyte T cells: Natural Killer T (NKT) cells share characteristics of both NK cells and T cells. They recognize glycolipid antigens like α-galactosylceramide presented by CD1d, an MHC class 1-like molecule. When activated, NKT cells secrete abundant pro-inflammatory cytokines and effector molecules involved in cell death (perforin, Fas-L, TRAIL). Increased tumor infiltration of NKT cells is associated in CRC with a better prognosis[16].

Human γδ T cells (γδ T cells) express a receptor to antigens combining a γ chain and a δ chain. This receptor can recognize different antigens usually in a non-MHC-restricted way, such as heat shock proteins or phosphorylated metabolites generated by tumor cells. γδ T cells been demonstrated to have a strong cytotoxic activity against tumor cells in CRC[17].

Macrophages: Tumor infiltrating macrophages (TIM) can be divided into two different subtypes with different roles in cancer[18]. M1 TIMs are intimately involved in innate immunity, as they target altered cells, produce pro-inflammatory molecules (IL-6, IL-12, IL-23 and TNFα) and promote adaptive immunity through increased expression of MHC and costimulatory molecules. They may also target tumor cells linked to antibodies because they express a receptor for immunoglobulin constant fragments (ADCC). Activated M2 TIMs are engaged in wound healing and can promote tumor progression through immunosuppressive cytokines (IL-10 and TGFβ). While infiltration by macrophages is generally a poor prognostic factor in different types of cancer, in CRC it seems to be associated with a better prognosis[19], suggesting that antitumorigenic properties dominate *in vivo*.

Adaptive immunity

A specific antitumor response is generated by the adaptive immune system, and in particular by αβ T cells. Briefly, the antigen-presenting cells (APCs), mainly dendritic cells (DCs), capture, process and present tumor antigens to CD4 T cells through MHC class Ⅱ or to CD8 T cells through MHC class Ⅰ. Activation of T cells requires 3 signals: (1) recognition of antigenic peptide presented by the APCs; (2) activation of costimulatory molecules (CD80/CD28, CD40/CD40L); and (3) recruitment of cytokines (IL-1, IL-2, IL-6, IL-12, IFNγ). Activated CD8 T cells can recognize and lyse tumor cells. Activated CD4 T cells modulate the antitumor immune response. They differentiate into different cell subgroups: The Th1 response allows secretion of cytokines that promote the antitumor response, as IL-2 or IFNγ, whereas the Th2 response favors tumor growth. The Th17 subset secretes large amounts of IL-17. Its role in the immune response against cancer is controversial. Finally, a subset of $CD4^+$ T cells called regulatory T cells (Tregs) and characterized by the expression of CD25 and Foxp3, inhibit the immune response and represent a widely described mechanism whereby the tumor can escape the immune system.

Tumor-associated antigens allow recognition of tumor cells by the immune system: Many cells and molecules are involved in immunosurveillance, they may be linked to the host or the tumor. First, tumor-associated antigens (TAAs) allow an immune response mediated by the humoral and cellular immunity. Several types of TAAs are expressed by the tumor. In CRC, the most frequent TAAs are normal self-antigens, expressed at low levels in normal cells and in embryonic tissues and at high levels in tumor cells. The most famous of them is the carcino-embryonic antigen (CEA), which is normally expressed in fetal tissue, and widely overexpressed in CRC[20]. If it has been shown initially that CEA can lead to a specific cytotoxic response[21], more recent works have shown that CEA may have an immunosuppressive role and that T cells of patients with CRC were not activated by the presentation of this antigen *in vitro*[22]. Other self-antigens are thought to be immunogenic in CRC, as Ep-Cam HER-2/neu[23], MUC-1 and p56. Immune responses against some neo-antigens, generated by mutations (tp53, Kras) or against antigen MAGE-3, belonging to the family of "cancer testis antigen" normally expressed by germ cells, have been less frequently identified[21].

TAAs, which likely play an important role in immunosurveillance, are also potential targets for immunotherapy in vaccination strategies.

Microsatellite instability CRC is associated to immunogenic TAAs: Microsatellite instability (MSI) is associated with CRC in patients with Lynch syndrome, but also with sporadic cancer, in particular in elderly patients, and is observed in 5% to 25% of CRC patients depending on tumor stage. MSI tumors are associated with a high density of tumor infiltrating lymphocytes (TILs)[24,25], and have a better prognosis than CRC without a microsatellite instability phenotype[26].

MSI induces frameshift somatic mutations within target genes harboring repeated sequences in their coding frame, including TGFβR2, which is mutated in 90% of

cases. These mutations lead not only to the inactivation of these target genes but also to the appearance of potentially immunogenic neoantigens. Indeed, disruption of the reading frame of TGFβR2 results in a new epitope (RLSSCVPVA) and in specific T cells to this epitope in tumors and peripheral blood of patients with MSI tumors[27]. Other MSI-associated mutations, as mutations of OGT[28], MSH3[29] caspase 5, ASTE1 and PTEN, have been shown to induce production of new immunogenic TAAs. Tougeron *et al*[30] studied 19 frequently mutated genes in CRC with MSI. In samples of stage Ⅱ or Ⅲ MSI tumors, an increased number of mutated genes was correlated with a high density of TILs. Mutations of ASTE1 and PTEN were particularly associated with increased lymphocyte infiltrate. These results suggest an important role of the immune response to specific neoantigens in CRC with MSI, and its potential involvement in the better prognosis of these tumors. Nevertheless, CRC associated with MSI may develop specific mechanisms to escape the immune system as for example particularly high levels of intratumoral Treg described in these patients[31]. Frameshift mutations can also induce inactivation of beta2-microglobulin leading to HLA class Ⅰ downregulation[32,33] though the association between HLA class Ⅰ downregulation and MSI is still controversial. Altogether, CRC associated with MSI could lead to a more intense immune response, but also to specific immunoregulatory phenomena, making them good candidates for immunotherapy.

Tumor infiltrate of memory CD8 T cells and CD45RO memory T cells may predict recurrence: The role of cytotoxic CD8 T cells has been widely studied in CRC. Tumor-infiltrating lymphocytes (TILs) are central to the antitumor immune response. The prognostic role of the immune response has been analyzed in a large cohort of resected patients.

Pagès *et al*[34] showed that the absence of pathological signs of early metastatic invasion (venous, lymphatic and perineural invasion) was associated with increased infiltrates of immune cells and increased levels of messenger RNA (mRNA) for products of Th1 effector T cells.

The density of TILs, characterized by CD3 immunostaining, has been reported to be more predictive of overall survival than all the usual histopathologic prognostic factors (*i.e.*, UICC-TNM classification)[35]. Five-year overall survivals in patients with high, intermediate or low $CD3^+$ TILs density were of 72.6%, 49.5% and 29.9%, respectively. In multivariate analysis, the density of TILs was still an independent prognostic factor, while TNM classification was no longer an independent factor after adjustment for the density of TILs.

Regarding phenotype, TILs were increased in tumors without signs of early metastatic invasion, especially memory CD8 T cells ($CD45RO^+$), ranging from early memory to effector memory T cells[34]. Finally, increased levels of $CD45RO^+$ correlated with increased overall survival and increased disease-free survival. In this large cohort, patients who had tumors with a high density of $CD45RO^+$ cells or with a low density of $CD45RO^+$ cells had a median disease-free survival of respectively 36.5 mo and 11.1 mo, and a median overall survival of respectively 53.2 mo and 20.6 mo ($P < 0.001$ for all comparisons). In multivariate analysis, the density of $CD45RO^+$ cells was still an independent prognostic factor.

Based on these results, an immune score based on immunostaining has been elaborated, considering 4 densities: density of $CD8^+$ T infiltrates in the center of the tumor (CT), in the invasive margin (IM), and density of memory $CD45RO^+$ cells in the CT and in the IM. This immune score was first studied in early-stage tumors (stages Ⅰ and Ⅱ)[36]. Patients with a high density of both $CD8^+$ and $CD45RO^+$ cells in both the CT and IM had a disease-free survival of 95.2%, compared with 25% in patients with a low density of both $CD8^+$ and $CD45RO^+$ cells in both regions. This immune score was validated in a cohort of 599 specimens of stage Ⅰ to Ⅳ CRC[37]. In this study, assessment of immune score was a better predictor of tumor recurrence (HR = 0.64; $P < 0.001$) than TNM classification. However, the immune infiltrate is highly heterogeneous in a tumor, and quantification is observer-dependent. To simplify and harmonize the quantification of immune infiltrate, automated quantification of $CD3^+$ cells can be used. Linear quantification of lymphocytes has been shown to be predictive of disease-free-survival in multivariate analysis with very good inter-observer reproducibility[38]. However, other teams have not confirmed these results yet and major information are lacking in this large retrospective series such as age, MSI status or the use of adjuvant therapy. Despite these promising results, there is still no immune quantification test in routine practice to use immune infiltrate to guide our therapeutic strategies. This underlines the difficulty to find a standardized and reproducible test that complies with daily practice. Such tests should be of particular interest for clinicians, especially for stage Ⅱ patients for whom the indication for adjuvant treatment is more controversial.

MECHANISMS OF IMMUNE SYSTEM ESCAPE IN COLORECTAL CANCER

Human leukocyte antigen class Ⅰ downregulation is associated with a poor prognosis

Expression of Human Leukocyte Antigen class Ⅰ (HLA-Ⅰ), the human MHC, class Ⅰ molecules is downregulated in more than 70% of colorectal tumors[39]. In a few cases there is complete loss of HLA-Ⅰ on tumor cells. Total loss of HLA-Ⅰ mainly results from beta2-microglobulin inactivation in MSI tumors and LMP7/TAP2 downregulation in MSI-negative tumors[33]. Downregulation can result from loss of HLA haplotypes due to chromosomal nondisjunction or mitotic recombination, loss of HLA locus expression, or allelic loss due to point mutations or partial deletions of HLA-Ⅰ genes. The prognostic significance of HLA-Ⅰ downregulation has

been reported in a large cohort of CRC cases[40]. Tumors with low expression of HLA- I were associated with a significantly shorter mean disease-specific survival (41 mo, 95%CI: 26-56) compared with tumors with high expression of HLA- I (68 mo, 95%CI: 63-74). Surprisingly, patients with a tumor with complete loss of HLA- I expression had a similar prognosis to those with high expression (mean disease-specific survival 60 mo, 95%CI: 50-69). This is possibly related to the high activity of NK cells against HLA- I -negative tumor cells. Killer inhibitory receptors, which are inhibitory receptors on NK cells, are dependent on MHC class I , then NK cells are activated in the absence of MHC class I . Tumor cells with downregulation but not complete loss of HLA- I expression could therefore avoid both T-cell- and NK-cell-mediated immune surveillance, and may be associated with a poor prognosis.

Induction of regulatory T cells

Induction of immunosuppressive cells is a major mechanism in escape from the host immune system. Tregs are characterized by expression of CD4, CD25, and Foxp3. In healthy individuals, role of Tregs is to prevent autoimmune disorders. In patients with cancer, Tregs could block the immune response against tumors through cytokine-dependent or cell-cell contact mechanisms. Tregs secrete immunosuppressive cytokines as IL-10 and TGFβ and immunosuppressive metabolites such as adenosine. The role of Tregs in cancer was first suspected from the observation of increased Tregs in peripheral blood and tumor tissue.

Strong Treg infiltration of tumors is generally associated with poor clinical outcome[41]. Elevated blood and tumor Treg numbers have also been described in CRC[42]. In some studies increased density of tumor-infiltrating Tregs is associated with a better prognosis[43], although in others elevated peritumoral numbers of CD4 and CD8 Tregs are associated with advanced-stage tumors and poorer overall survival[44]. This difference may be related to the heterogeneity of methods for characterization and quantification of Tregs and the use of more reliable techniques such as flow cytometry have shown the deleterious role of Tregs. In murine models of CRC, systemic removal of Tregs using anti-CD25 antibody results in tumor rejection and in improved vaccine-induced antitumor T-cell responses[45,46]. In human models, *in vitro* Treg depletion from peripheral blood of patients with CRC induces CD4 and CD8 T-cell responses against tumor-associated antigens[47,48]. Altogether, there is considerable evidence that Tregs are associated with a poor outcome in CRC.

Accumulation of Treg in tumors could be explained by several mechanisms[49]. The first mechanism is the conversion of conventional $CD4^+$ T cells into Treg in response to various signal, especially secreted or membrane TGFβ. Tumors can also induce a preferential recruitment of Treg in tumors through the production of chemokines such as CCL17, CCL22 and CCL28[50,51]. VEGF-A secreted by tumor in response to hypoxia seems also to play a crucial role in tumor-induced Treg. VEGF-A inhibits maturation of DC. Immature DC, which can express TGFβ, can favor the conversion of conventional T cells into Treg[52,53]. VEGF-A can also directly promote expansion of Treg through VEGFR-2 expressed on the cell membrane of a Treg subgroup[54]. Recent data suggest that the number of intratumoral $FOXP3^+$/$VEGR\text{-}2^+$ Tregs is more predictive of recurrence and survival than the number of $FOXP3^+$ alone in CRC[55].

Other escape mechanisms

Other escape mechanisms are suspected in CRC (Figure 1). B7-H1, or PD-L1, is a costimulatory molecule known to regulate T cell function negatively by interaction with PD-1. B7-H1 is strongly expressed in CRC[56] and is associated with poor prognosis[57]. B7-H1 may thus play an important role in tumor cell proliferation, apoptosis, migration and invasion. Other molecules, such as CTLA-4, are involved T lymphocytes inhibition. CTLA-4 is expressed on the surface of T lymphocytes, and its ligands, CD80 and CD86, are expressed on the surface of APCs. Expression of these molecules, called "immune checkpoints", are important mechanisms of inhibition of antitumor immune response. Recently some monoclonal antibodies targeting these molecules (PD1, CTLA-4) have shown more than promising efficacy results in solid neoplasia such as melanoma and others[58-60].

Myeloid-derived suppressor cells (MDSC) are immunosuppressive cells. As Tregs, they contribute to the immune tolerance by inhibiting the function of $CD8^+$ T cells. The prognostic value of MDSC is not well known, but they are thought to be deleterious, as elimination of MDSC in mouse tumor models was shown to enhance antitumor responses, resulting in tumor regression[61].

IMPACT OF ANTICANCER TREATMENTS ON IMMUNITY IN COLORECTAL CANCER

Chemotherapy induces immunogenic cell death

Some cytotoxic chemotherapy are known to induce immunogenic cell death. In CRC murine models and human tissues, oxaliplatin- but not cisplatin-based chemotherapy can trigger pre-apoptotic calreticulin exposure and the post-apoptotic release of high-mobility group box 1 protein (HMGB1), two signals which are required for immunogenic cell death[62]. DCs have several receptors for HMGB1, including Toll-like receptor 4 (TLR4). In a murine model with CT26 tumor cells, oxaliplatin-treated dying cells failed to elicit an antitumor immune response in TLR4-deficient mice, while *TLR4*+/+ controls were protected against rechallenge with the same cancer cells. Twelve to 14% of Caucasian patients present the loss-of-function allele of TLR4. In patients from the FFCD 2000-05 randomized trial (Ducreux lancet Oncol) with stage Ⅳ CRC and treated with an oxaliplatin-based combination, the TLR4 loss-of-function allele was associated with reduced progression-free and overall

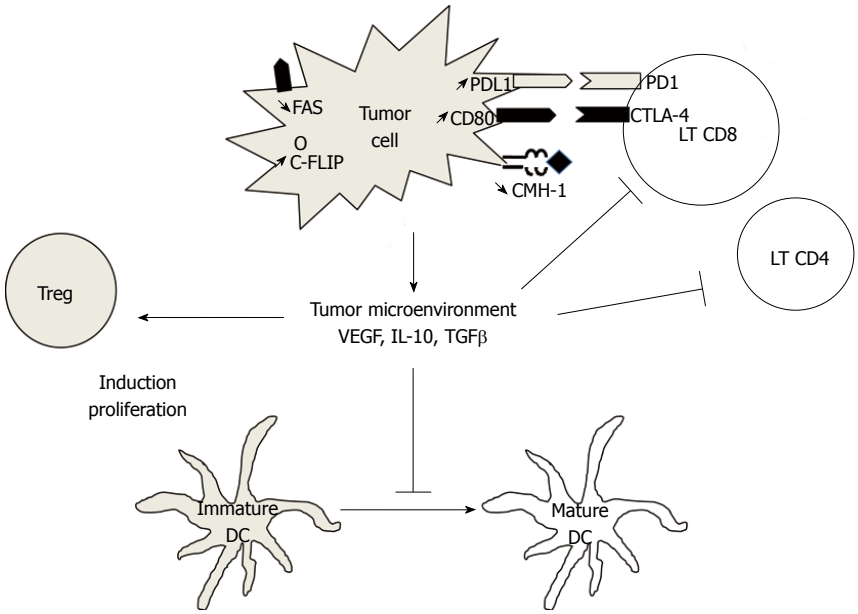

Figure 1 Major mechanisms of tumor immune escape. Tumor cells can induce immunosuppression by different pathways: (1) tumor cells can secrete immunosuppressive cytokines as vascular endothelial growth factor (VEGF), interleukin-10 (IL-10) or transforming growth factor-β (TGFβ). These molecules contribute to blockade of maturation of dendritic cells (DC) and then induce regulatory T cells (Treg) rather than lymphocytes T (LT) $CD4^+$ or $CD8^+$ cells. VEGF could promote directly induction and proliferation of Treg; (2) Tumor cells down-regulate expression of CMH-1; (3) Tumor cell express inhibitory molecules as PDL1 or CD80, and induce exhaustion of LT; and (4) Tumor cells can evade apoptosis by inducing anti-apoptotic molecules such as C-FLIP, or by down-regulating the expression of death receptors such as FAS.

survival, as compared with patients carrying the normal TLR4 allele[63]. This allele, however, was not associated with disease-free survival in another cohort of patients who underwent surgery for CRC stage Ⅱ and who did not receive chemotherapy, suggesting that TLR4 is predictive of chemotherapy effectiveness, but is not a prognostic factor.

Other check-points, such as the P2X7 receptor (P2RX7), which has a high affinity for ATP released by dying tumor cells and carried by DCs, are required for the anticancer immune response induced by chemotherapy and could modulate susceptibility to treatments[64].

Others immune mechanisms could be induced by cytotoxic chemotherapy. It has been shown in murine model that 5-fluorouracil could lead to a decrease of MDSC in the spleen and tumors *in vivo*, combinate to a T cell-dependent antitumor responses[61], but the therapeutic impact is not well established.

All these data suggest that the immune system may participate to the therapeutic effect of chemotherapy in CRC but should be confirmed in future works prospectively dedicated to this question.

Anti-VEGF therapy inhibits Treg expansion

As seen above, tumors can induce immunosuppressive cell populations such as Tregs. It is now well established that antiangiogenic agents decrease Treg numbers in blood and tumors. In peripheral blood of patients with renal carcinoma and different models of tumor-bearing mice, sunitinib reduces Treg numbers, and the decrease in Tregs is associated with overall survival in patients series[65,66]. In a recent study, we investigated the immunomodulatory effect of antiangiogenic agents in a mouse model of colon cancer[54]. Tregs decrease to their physiological level after treatment with sunitinib or VEGF-A antibody. However, after masitinib treatment, a multi-target tyrosine kinase inhibitor close to sunitinib but not targeting the VEGFR, Tregs were not reduced. VEGFR-2- but not VEGFR-1-specific blockade led to the same results. These results suggest that targeting the VEGF-A/VEGFR-2 pathway is sufficient to decrease Tregs in murine models of CRC. Bevacizumab directly inhibits this pathway and has been widely used in CRC since 2004[67]. In patients with metastatic CRC, we found that bevacizumab inhibited Treg accumulation and proliferation in peripheral blood. Antiangiogenic agents could act on other immunosuppressive cells, such as myeloid-derived suppressor cells and exhausted T cells[68]. Once again it is difficult to argue that the immunomodulating effect of bevacizumab in patients with CRC has an impact on its therapeutic efficacy. But in the future Tregs monitoring could help to predict response to bevacizumab. Furthermore this immunomodulatory effect of anti-angiogenic agents could be used to potentiate immunotherapeutic strategies.

Activity of cetuximab may depend in part on ADCC

Monoclonal antibodies used in therapeutics act on specific receptors to inhibit growth pathways. Some may also induce immune phenomena related to the characteristics of natural antibodies. In particular, cetuximab (chimeric IgG1 monoclonal antibody) binds epidermal growth factor (EGFR) and is used in RAS wild type metastatic CRC. It has been suggested that cetuximab, in ad-

dition to direct inhibition of EGFR, may act *via* ADCC. ADCC allows the antitumor innate immune response but can also trigger the adaptive immune response[69]. *In vivo*, addition of CpG, a TLR9 agonist able to activate DCs, increases immune response to cetuximab and its therapeutic efficacy.

Single nucleotide polymorphisms (SNPs) in the coding region of FCγR2A or FCγR3A have been reported to correlate with responses to cetuximab. The role of FCγR2A H/H or FCγR3A V/V genotypes is especially controversial[70]. Three studies in metastatic CRC showed a beneficial effect of FCγR3A V/V polymorphisms, and two of these studies also showed a beneficial role of FCγR2A H/H polymorphism. These polymorphisms were associated with better progression-free survival or objective response rate in patients treated with cetuximab. However, three other studies reported that FCγR3A V/V polymorphism was associated with shorter survivals in patients treated with cetuximab.

IMMUNOTHERAPY IN CRC

As in other cancers, immunotherapy could represent a step forward in the treatment of CRC.

Several strategies are being investigated in the treatment of CRC. They are presented in Figure 2. Some have already been tested in clinical trials or are currently being tested in ongoing trials (Table 1).

Association of chemotherapy and nonspecific immunotherapy

Nonspecific immunotherapy consists of stimulation of host immunity with cytokines such as interferon (IFN), interleukins or GM-CSF. A phase Ⅱ study tested the combination of GM-CSF, gemcitabine and FOLFOX (GOLFIG regimen) in 46 patients in first- to third-line treatment[71,72]. This regimen was safe and active in pretreated patients. Prolonged survival and time to progression were associated with signs of autoimmunity and with an increase in memory T-cells and a decrease in Tregs in the peripheral blood of patients. A phase Ⅲ study compared GOLFIG with FOLFOX[73]. The study was ended prematurely as an intermediate analysis showed significant superiority of GOLFIG over FOLFOX chemotherapy in terms of response rate (59.3% *vs* 34.4%, $P = 0.0001$) and progression-free survival (12.4 mo *vs* 7.9 mo, HR = 0.64, $P = 0.0105$). Autoimmunity signs, tumor infiltration by Tregs and central memory T cells were independent predictive markers of efficacy in this work.

Vaccination trials

Vaccination against tumor antigens: Few phase Ⅱ trials involving antigen vaccination have been reported in the setting of CRC. Immunization with β-human chorionic gonadotropin (βHCG) peptide vaccine in mostly pretreated patients with metastatic CRC induced anti-hCG antibody in 56 of the 77 patients. High levels of antibody were associated with significantly longer survival[74]. Other adjuvant vaccinations with antigen were studied. Immunization with CEA after curative resection of hepatic metastases did not improve 2-year recurrence-free survival[75]. A pilot study of adjuvant vaccination with a mutant RAS peptide in KRAS mutated stage Ⅱ and Ⅲ CRC induced a specific immune response with increased IFN-γ mRNA expression in 4 out of 7 patients and was well tolerated[76]. Several ongoing phase Ⅰ/Ⅱ studies are studying antigen vaccines using various peptides, as mucinous glycoprotein 1 (MUC, L-BLP25), MSI, or HER2neu.

Vaccination with autologous tumor cells: Since 1992 active specific immunotherapy (ASI), consisting of immunization with irradiated autologous tumor cells as adjuvant therapy, has led to a few phase Ⅲ trials. The first strategy of ASI was to use Newcastle disease virus-infected autologous tumor cell vaccine after resection of hepatic metastases with curative intent[77]. The second strategy used an autologous tumor cell BCG vaccine (OncoVax) in stage Ⅱ or Ⅲ CRC[78,79]. In the 3 studies no significant benefit was observed in the overall population, but some subgroups appeared to benefit from vaccination more than others, especially colon cancer (*vs* rectal) and stage Ⅱ cancers (*vs* stage Ⅲ). Patients with stage Ⅱ CRC treated with OncoVax had a 5-year recurrence rate of 21.3% *vs* 37.7% in the control group, leading to a significantly better 5-year recurrence-free survival ($P = 0.009$), although there was no difference in stage Ⅲ patients[80]. These results have not yet been confirmed and should lead to a pivotal phase Ⅲ trial.

Dendritic cell-based vaccination: A significant improvement in antitumor vaccination is provided by vectorization of antigens, in particular with DCs[81]. Pilot studies have also proposed DC-based vaccination in CRC, using DCs loaded with a single antigen[82-84], two antigens[85] or multiple antigens[86-88] with a good safety profile. In some cases autologous DCs or antigens are used, making the procedure labor-intensive and costly. This promising strategy is one of the most used in ongoing immunotherapy clinical trials in CRC, but other vectorization strategies, such as synthetic vectors, could be used in the future[89] and could be more efficient and simpler than those with DCs.

Adoptive cell therapy: Adoptive cell therapy (ACT) is mostly used in melanoma. Briefly, T cells are collected from the tumor, draining lymph nodes or peripheral blood, and are activated and expanded *in vitro*. Autologous T cells are then administered intravenously to the patient. To optimize the activity of ACT, some authors have tried lymphodepletion of the host, optimized cytokine cocktails and selection of $CD8^+$ T cell clones with higher affinity for tumor cells/antigens. ACT with T cells from patient lymph nodes has been tested in 16 patients with stage Ⅱ to Ⅳ CRC[90]. ACT was well tolerated

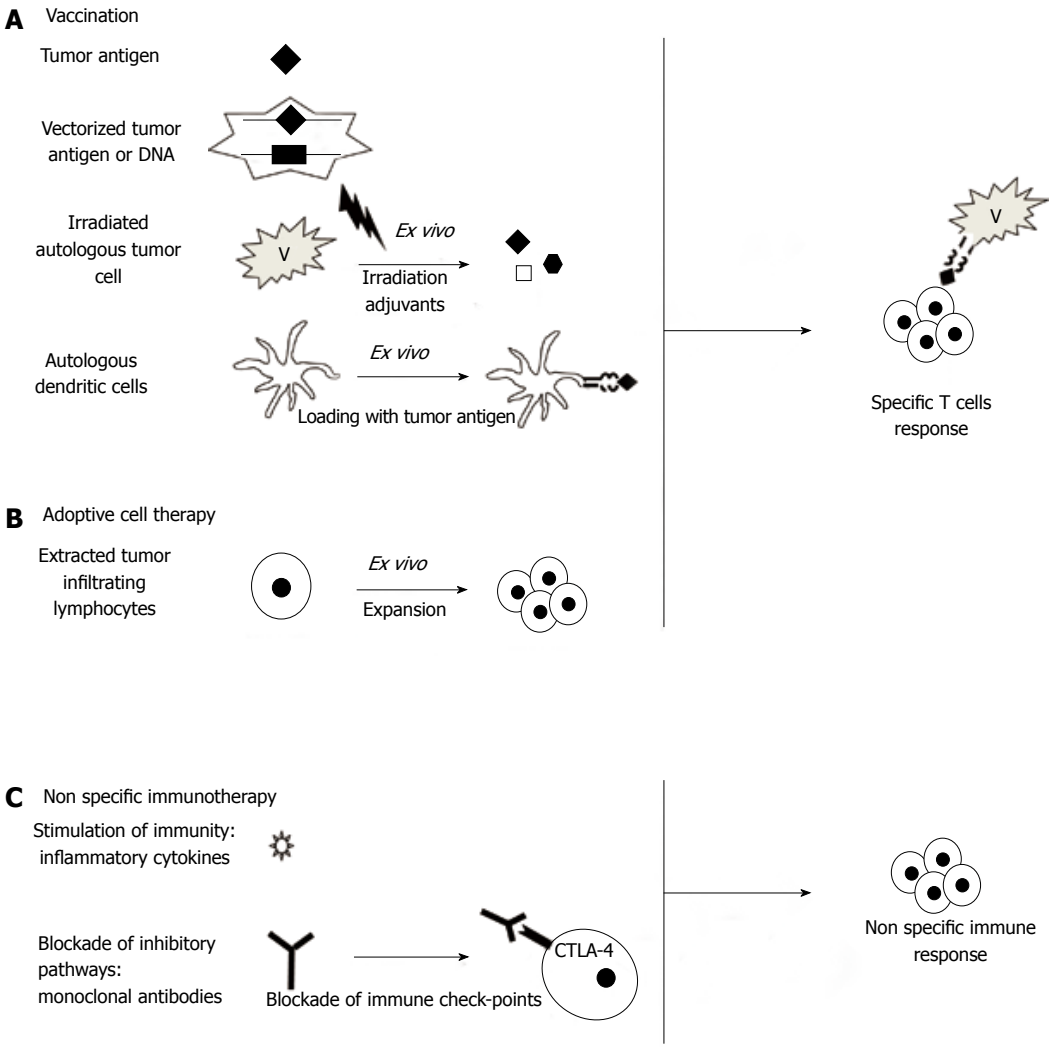

Figure 2 Main current immunotherapy strategies. A: The goal of vaccination is induction of a specific immunization against tumor antigens. The agent used for immunization can be either a single antigen, administered systemically or directly vectorized path, or a combination of antigens obtained from irradiated tumor cells. Finally, the antigen can be loaded *ex vivo* in autologous dendritic cells; B: Adoptive cell therapy is based on the *ex vivo* expansion of immune cells of the host in the presence of tumor cells, allowing the expansion of specific clones. This is mainly T cells but could also involve natural killer cells or a combination of immune cells; C: The non-specific stimulation of the immune system can be obtained by the administration of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor, or by blocking inhibitory pathways. Ipilimumab is a monoclonal antibody blocking cytotoxic T lymphocyte-associated antigen 4 (CTLA-4).

in all cases with no side effects, and allowed a complete response in 4 out 9 patients with metastatic disease.

Similarly, autologous genetically engineered T cells with high-avidity CEA-specific T cell receptor have been used in CRC[91]. In a phase I study, 3 patients were treated and decreased serum CEA levels were observed, but all patients developed severe colitis. Genetically engineered T cells expressing chimeric antigen receptors targeting HER2 also led to severe toxicity in a patient with CRC[92]. Similar strategies, such as allogenic lymphocytes and autologous NK therapy, are currently being tested in phase I and II studies.

CONCLUSION

The immune system plays a major role in the eradication of tumor cells, but is bypassed by the tumor at the clinical expression phase. Various antitumor immune mechanisms are inhibited by efficient escape mechanisms. The treatments currently used in CRC (cytotoxic chemotherapy, anti-EGFR antibodies, antiangiogenic molecules) are associated with immunomodulating effects shown *in vitro* and *in vivo*. However, their clinical impact has not been well evaluated. In some cases the immune escape mechanisms are associated with an aggressive phenotype. In these cases classic treatments clearly fail, and immunotherapeutic approaches is a seducing alternative to try to improve the prognosis of these patients in the future. Several approaches can be considered. First, nonspecific immunotherapy that may use immunostimulatory molecule (GM-CSF, IL-2, IL-7) or inhibit immunosuppressive mechanisms (Treg depletion, anti-PDL1, anti-CTLA4). Second, the purpose of specific immunotherapy is the induction of a specific antitumor immune response. Various vaccination strategies, with peptide, antigen, DNA combined with vectorization techniques, could lead to

Table 1 Ongoing clinical trials, according to National Cancer Institute registration, using immunotherapy, according to strategy

Principle	Phase	Specificity	Registration number
(A) Peptide vaccine		Targeted peptide(s):	
	I	Ras mutated	NCT01322815
		MUC-1	NCT01556789
		HER2/neu	NCT01730118
	I / II	Survivin	NCT00108875
		Frame shift peptides (MSI)	NCT01461148
		Nor-MDP	NCT01376505
	II	MUC-1	NCT01462513
(B) Whole cell cancer vaccine		Characteristic of cancer cells:	
	I / II	Allogenic cancer cell	NCT00722228
			NCT00656123
(C) DC-based therapy		Characteristic of DCs:	
	I / II	Autologous DCs intratumoral injection	NCT01882946
		Loaded with Frame shift antigens (MSI)	NCT01885702
		CEA-pulsed DCs^{+} IL-2	NCT00154713
	II	Autologous DCs	NCT01348256
			NCT01413295
(D) Inhibition of immunoregulation		Immunomodulation strategy:	
	I / II	Treg depletion	NCT00986518
		Anti-CTLA4 + local radiation therapy	NCT01769222
(E) Non specific immunostimulation		Immunostimulatory agent	
	I / II	Recombinant vaccinia virus	NCT01394939
		IFN, Celecoxib + combination of chemokines	NCT01545141
	II	IL-7	NCT01339000
		Heat killed whole cell mycobacterium	NCT01539824
	III	PGG beta-glucan: binding to neutrophils	NCT01309126
(F) Cell therapy		Characteristic of cells	
	II	Allogenic activated lymphocytes	NCT00149006
			NCT00855452
		Autologous TILS + lymphocyte depletion	NCT01174121
		Engineered autologous anti-ESO-1 lymphocytes	NCT00670748
		Engineered autologous anti-CEA lymphocytes	NCT01723306
	NS	Autologous natural killer T cells	NCT01801852

Several strategies are used in clinical trials of immunotherapy: (A) Vaccination with direct injection of one or more peptides; (B) Immunization using whole irradiated tumor cells; (C) Vaccination using autologous dendritic cells (DCs) and/or charged DCs with one or more antigens; (D) Inhibition of immunoregulatory mechanisms; (E) Nonspecific stimulation of the immune system; (F) Adoptive cell therapy using tumor infiltrating lymphocytes (TIL) or lymphocytes from peripheral blood, possibly reworked to target specific antigens. Source: http://clinicaltrials.gov/. IL: Interleukin; IFN: Interferon.

the development of effective vaccines, particularly in the adjuvant setting. ACT with T cells or NK cells is a labor-intensive procedure, but advances in genetic engineering raise hope for such treatments. Finally, nearly 40 phase I to III clinical trials testing immunotherapy in CRC are ongoing. This will probably lead in the near future to consider one or a combination of these different strategies in our therapeutic armamentarium to fight CRC.

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TOPIC HIGHLIGHT

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Gastro-intestinal toxicity of chemotherapeutics in colorectal cancer: The role of inflammation

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Abstract

Chemotherapy-induced diarrhea (CID) is a common and often severe side effect experienced by colorectal cancer (CRC) patients during their treatment. As chemotherapy regimens evolve to include more efficacious agents, CID is increasingly becoming a major cause of dose limiting toxicity and merits further investigation. Inflammation is a key factor behind gastrointestinal (GI) toxicity of chemotherapy. Different chemotherapeutic agents activate a diverse range of pro-inflammatory pathways culminating in distinct histopathological changes in the small intestine and colonic mucosa. Here we review the current understanding of the mechanisms behind GI toxicity and the mucositis associated with systemic treatment of CRC. Insights into the inflammatory response activated during this process gained from various models of GI toxicity are discussed. The inflammatory processes contributing to the GI toxicity of chemotherapeutic agents are increasingly being recognised as having an important role in the development of anti-tumor immunity, thus conferring added benefit against tumor recurrence and improving patient survival. We review the basic mechanisms involved in the promotion of immunogenic cell death and its relevance in the treatment of colorectal cancer. Finally, the impact of CID on patient outcomes and therapeutic strategies to prevent or minimise the effect of GI toxicity and mucositis are discussed.

Key words: Chemotherapy; Diarrhea; Side-effects; Immunogenic cell death; Pro-inflammatory cytokines

Core tip: Many new drugs are available for use in the treatment of colorectal cancer, resulting in improved prognosis, but also more frequent and severe side-effects. In order to implement complex chemotherapy regimens most effectively, a greater understanding of the underlying mechanisms of associated toxicities are required. Different chemotherapeutic agents activate a diverse range of pro-inflammatory pathways culminating in distinct histopathological changes in intestinal mucosa. However, inflammation also has beneficial effects; enhancing anti-tumor immunity. A better understanding of how to manage the gastrointestinal side-effects of chemotherapy allowing for optimal dosing and induction of immunity will further improve outcomes in colorectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is the one of the most common forms of cancer worldwide and is the fourth most common cause for cancer related death[1]. While its incidence is continuing to rise in developing countries, developed countries such as the United States are observing a falling trend in CRC, likely secondary to screening[2]. Prognosis of CRC in the developed countries has also improved, with CRC specific mortality falling over the past 20 years[3]. The reasons behind this are multi-factorial and include earlier diagnosis and increased access to better oncological care. Advancements made in systemic chemotherapy for CRC and the development of novel biological agents have contributed to increased patient longevity; by preventing recurrence of disease in non-metastatic cases, and by down-staging or preventing disease progression in metastatic cases[4]. However, with this progress comes an increased incidence of drug toxicities and side-effects. To obtain the maximum benefit of new combination chemotherapy regimens, a better understanding of side effects and patient management is required.

Gastrointestinal toxicity is one of the most commonly encountered side effects experienced during systemic therapy for CRC[5]. Chemotherapy induced diarrhea (CID) has been reported to affect 50% of CRC patients receiving 5-fluorouracil (5-FU) as single agent and severe CID can develop in up to 40% of patients receiving combination chemotherapy[6]. CID is one of the major causes of dose limiting serious toxicity in chemotherapy regimens containing 5-FU. Chemotherapy agents exert toxic damage on the gastrointestinal (GI) epithelium which is at least partly mediated by activating the inflammatory cascade. Herein we review the mechanisms that are involved in GI toxicity during chemotherapy for CRC and their potential effect on cancer cells; by triggering immunogenic cell death, which in turn may have an impact on cancer relapse and survival.

CHEMOTHERAPY USE AND ASSOCIATED TOXICITY

5-FU is the main backbone agent used in systemic chemotherapy for CRC. When used as a single agent or as combination therapy with Oxaliplatin or Irinotecan in adjuvant chemotherapy there is evidence to show that a reduction in relapse by up to 33% can be achieved[7]. 5-FU based combination therapies have also shown efficacy in advanced CRC by improving progression free survival[8]. CID is a common side effect encountered during 5-FU based chemotherapy. It has been reported that 50%-80% of patients receiving 5-FU based adjuvant therapy for CRC develop CID of any grade; while grade 3 or 4 CID occurred in up to 30% of patients in clinical trials (Table 1). GI toxicity from 5-FU is influenced by several factors with different chemotherapy regimens generating varying incidences of CID. 5-FU given as a short infusion appeared to be better tolerated with less GI side effects as compared to 5-FU given as a bolus[9]. Capecitabine, an oral fluoropyrimidine, has shown similar efficacy to intravenous 5-FU in clinical trials with better safety profile and less diarrhea though there was no difference in the number of reported cases of severe (Grade Ⅲ or Ⅳ) CID[10].

Combination therapy has shown better efficacy and survival compared to single agent 5-FU therapy[11]. However such combinations enhance treatment related toxicities, including CID. This is especially evident in combination therapy of intravenous 5-FU and Irinotecan; as both 5-FU and Irinotecan have been shown to have direct toxic effects on the intestinal mucosa[12]. In trials where bolus Irinotecan were given with weekly bolus 5-FU and Leucovorin (IFL regimen) for CRC, an unacceptably high rate of GI toxicity and mortality were observed[13,14]. This toxicity is ameliorated somewhat with another regimen, whereby short term infusional 5-FU is administered together with Irinotecan every other week (FOLFIRI regimen); with reported grade 3 or 4 diarrhea incidence of around 14 percent[15].

Similarly Oxaliplatin combined with intravenous 5-FU has shown increased rates of GI toxicity. Short infusional 5-FU in combination with Oxalipatin (*e.g.*, FOLFOX regimen) was noted to be better tolerated than combination therapy with weekly bolus 5-FU (*e.g.*, FLOX regimen) in terms of CID; highlighting the importance of drug scheduling of 5-FU in the development of GI toxicity[16]. The mode of fluoropyrimidine administration also seems to have an impact on the toxicity profile in combination therapy. Capecitabine combined with Oxaliplatin (XELOX regimen) for treatment of metastatic CRC has shown similar efficacy but reduced incidence of severe diarrhea was noted compared to FOLFOX (14% *vs* 24%)[17]. In contrast, Capecitabine combined with Irinotecan (XELIRI) resulted in higher rates of severe CID compared to FOLIRI during treatment for metastatic CRC; indicating that toxicity profiles between different forms of fluoropyrimidine administration cannot be automatically assumed when combined with other drugs[15].

There is now increasing use of targeted therapies in the management of metastatic CRC[18]. While these agents seldom cause severe CID alone; they could further potentiate GI toxicity when given in combination with standard chemotherapy[19]. Therefore continued pharmaco-vigilance for GI toxicity is needed as the complexity of systemic chemotherapy of CRC rises with new treatment combinations.

MECHANISMS UNDERLYING CHEMOTHERAPY INDUCED MUCOSITIS

The manifestations of chemotherapy induced GI toxicity have been mainly attributed to the disruption of the mucosal barrier which lines the whole alimentary tract caused by the treatment; termed "mucositis". Previously thought as just an epithelial phenomenon when cells are exposed to chemotoxic agents or radiotherapy; it is in-

Table 1 gastrointestinal toxicity profile of fluoropyrimidine based chemotherapy used in colorectal cancer

Regimen	Patient setting	CID overall	CID grade 3/4	Oral mucositis overall	Oral mucositis grade 3/4	Ref.
Flouropyrimidine monotherapy						
5-FU/LV bolus	Adjuvant	79%	21%-30%	28%	1%-8.1%	[93]
5-FU/LV infusion	Adjuvant	-	4%	-	2%	[9]
Capecitabine oral	Adjuvant	46%	11%	22%	2%	[10]
Combation therapy with Oxaliplatin/Irinotecan						
FLOX	Adjuvant	-	38%	-	-	[16]
FOLFOX	Adjuvant	56.3%	10.8%	41.6%	2.7%	[94]
XELOX	Adjuvant	60%	19%	21%	< 1%	[95]
FOLFOX	Advanced CRC	46%	5%	30%	1%	[96]
XELOX	Advanced CRC	50%	14%	28%	2%	[17]
FOLFIRI	Advanced CRC	63%	10%	35%	1%	[96]
XELIRI	Advanced CRC	-	47.5%	-	-	[15]

CID: Chemotherapy-induced diarrhea; CRC: Colorectal cancer; 5-FU/LV: Intravenous 5-fluorouracil and leucovorin; FLOX: Bolus 5-FU and oxaliplatin; FOLFOX: Infusional 5-FU and oxaliplatin; XELOX: Oral capecitabine and oxaliplatin; FOLFIRI: Infusional 5-FU and irinotecan; XELIRI: Oral capecitabine and irinotecan.

creasingly recognized that the pathobiology of mucositis is complex involving the mucosal immune system with an important role played by pro-inflammatory cytokine release. The clinical effects of mucositis vary according to anatomical site. Oral mucositis and mucositis affecting the upper GI tract causes painful ulcerations and dysphagia. Mucositis of the small and large bowel results in abdominal cramps, bloatedness and diarrhea[20].

The five stage model proposed by Sonis *et al*[21] is very useful in explaining the basic pathobiology of mucositis. In brief, the model comprises of 5 phases occurring sequentially; (1) initiation; (2) up-regulation and message generation; (3) signaling and amplification; (4) ulceration and inflammation; and (5) healing phase[22]. The initiation phase occurs when GI mucosa are exposed to cytotoxic agents resulting in cellular DNA damage and cell death mainly through the generation of oxidative stress and reactive oxygen species (ROS). ROS directly induce tissue injury and trigger a cascade of inflammatory pathways.

During the second phase, significant up-regulation of inflammatory mediators is observed and nuclear factor kappa-B (NF-κB) is thought to be pivotal in this process. Once activated by chemotherapy and ROS, NF-κB acts to induce gene expression and production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, which in turn lead to tissue injury and apoptosis. NF-κB also causes up-regulation of gene expression of adhesion molecules and cyclooxygenase-2 (COX-2), with consequent angiogenesis.

During the third phase, a flood of pro-inflammatory mediators amplifies the whole inflammatory process *via* positive feedback loops, thus prolonging tissue injury. During this phase the process mainly occurs at the level of the submucosa and basal epithelium, therefore obvious damage to mucosal integrity is not observed clinically although the tissue biology is altered.

The fourth phase of mucositis is characterized by ulcerations and atrophic changes of the GI mucosa as a culmination event of tissue injury and stem cell death. GI epithelial integrity is destroyed and its function impaired. Patients are generally symptomatic during this phase. Bacterial colonization at the mucosa ulcers further induces inflammation by stimulating infiltration and activation of macrophages. Finally, the healing phase leads to renewal of epithelial proliferation and differentiation of the GI mucosa. This process occurs at approximately two weeks post chemotherapy and is also marked by angiogenesis implicating the importance of COX-2 in the process[22].

The histopathological changes associated with GI mucositis are well described. In humans, Keefe *et al*[23] studied patients undergoing chemotherapy with sequential duodenal biopsies pre and post treatment. They found that an increase in apoptosis was the first histological effect to be noted, with a seven-fold increase in apoptosis in intestinal crypts at day one post treatment. Reduction of intestinal villous area, crypt length and crypt proliferation then followed and the maximal effect was observed 3 d post treatment.

ANIMAL MODELS FOR STUDYING THE MECHANISM OF MUCOSITIS

Animal models have also been developed for the study of GI mucositis. Pertaining to chemotherapeutic agents used in CRC, Irinotecan and 5-FU based murine models are extensively researched and published.

IRINOTECAN HYDROCHLORIDE

Irinotecan hydrochloride (or CPT-11) exerts its antitumor effect by inhibiting DNA topoisomerase I[24]. The active metabolite, SN-38, induces irreversible DNA damage to tumor cells and its accumulation in the intestinal mucosa is thought to be responsible for enterotoxicity. SN-38 is glucuronidated in the liver to a non-toxic form (SN-38G) and excreted in the bile. Diarrhea is one of the major side effects of Irinotecan and patients encounter

two distinct types of diarrhea. Irinotecan induced early onset diarrhea occurs during or within several hours of administration and is cholinergically mediated and can therefore be prevented or ameliorated with atropine or anti-cholinergic agents. A second form of late onset diarrhea, which is not cholingerically mediated, ensues and mainly resulting from direct toxicity to GI mucosa in addition to other factors such as GI dysmotility[25].

Araki *et al*[24] reported that daily intraperitoneal injection of Irinotecan for 5 d causes severe diarrhea in athymic mice and haemorrhagic colitis by 7 d post treatment. Gibson *et al*[26] studied the histopathological changes associated with late onset irinotecan induced diarrhea on dark agouti (DA) rats by administrating daily intraperitoneal irinotecan for 2 d at varying doses and then examined the rats at fixed time points up to 96 h. They found irinotecan causes diarrhea by inducing apoptosis and hypoproliferation both in the small and large intestine. Additionally reduction in goblet cell numbers and mucin hypersecretion were noted in the colonic mucosa contributing to diarrhea. Similarly another study using a mouse model of irinotecan induced diarrhea found increased apoptosis together with structural changes in the GI mucosa and concluded that both malabsorption and mucin hypersecretion are likely to be at play[27].

Using the DA rat model, Bowen *et al*[28], 2007 looked at alterations in gene expression in Irinotecan induced diarrhea using mircoarray analysis and RT-PCR. They found multiple genes implicated in the mitogen-activated protein kinase (MAPK) signaling pathway were differentially regulated following Irinotecan treatment. These included IL-1 receptor, caspases, protein kinase C and dual-specificity phosphatase 6. Caspase-1 expression in jejunal tissue and was significantly increased 6 h after treatment and they conclude that GI damaged noted in chemotherapy utilizes the caspase cascade pathway, much like radiation induced damage and may be a potential target to prevent apoptosis following treatment. Logan *et al*[29] demonstrated with this model that in addition to histological changes noted in the GI mucosa, tissue staining for NF-κB, TNF-α, IL-1β and IL-6 were enhanced when compared to controls and peaked at between 2 and 12 h post administration. This provides further support for the role of pro-inflammatory cytokines in the pathogenesis of GI mucositis and the central role of NF-κB in the process. A mouse model of delayed diarrhea from Irinotecan also showed increase in pro-inflammatory cytokines and myeloperoxidase in intestinal tissue[30]. Additionally, they reported that thalidomide (known to have anti-TNF effects) and pentoxifylline (a methylxanthine derivative which reduces the expression of proinflammatory cytokines) decreased inflammatory infiltration and lesions induced by Irinotecan in treated mice. They conclude that cytokines regulate and amplify the immune response resulting in the injury and complications observed and that TNF-α, IL-1β and KC, (a mouse ortholog of human IL-8) are important mediators of this process. Using inducible nitric oxide synthase (iNOS) knock-out mice, the same group demonstrated that iNOS has an important role in the pathogenesis of mucositis. Furthermore, Infliximab, a monoclonal antibody against TNF-α, led to the reduction of intestinal expression of iNOS in irinotecan treated mice. Thus, suggesting that inflammatory cytokines and nitric oxide are among the main drivers of tissue damage in this model of mucositis[31].

5-FU

5-FU is an antimetabolite that acts on the enzyme thymidylate synthetase which in turn block DNA synthesis; thereby exerting its anti-tumor effects. Recognized common toxicities from 5-FU therapy include diarrhea and myelosuppression[32]. Several animal models exist for investigation of 5-FU associated toxicity and there is an increasing body of literature looking specifically at the mechanistic action of intestinal mucositis caused by 5-FU. Earlier studies conducted in mice models established the microscopic features of GI mucositis in 5-FU toxicity[33]. Pritchard *et al*[34] demonstrated in a murine model that 5-FU induced loss of crypt and villous cellularity through apoptosis and inhibition of cell cycle progression. Moreover these changes were significant reduced in p53 null mice; indicating that this process is p53 dependent.

Logan *et al*[12] examined GI mucositis in DA rats after a single administration of 5-FU (150 mg/kg intraperitoneally). They noticed shortening of crypt length, blunting and fusion of villi, enterocyte hyperplasia and increased apoptosis in the small intestine while decreased crypt length and increased apoptosis were noted in the colon. Interestingly immunochemistry on mucosal tissue of these rats showed elevation of TNF-α and IL-1β levels but no significant increased staining for NF-κB and IL-6. This indicates that apoptotic and inflammatory changes in 5-FU-induced mucositis may be secondary to pathways independent of NF-κB. In contrast, a study utilizing transcriptomic analysis was able to show that 1614 genes were upregulated in 5-FU-induced mucositis and that expression network revealed NF-κB as the central molecule in the process[35]. Furthermore bioluminescence imaging of transgenic mice showed increased NF-κB activity in the whole body 2 d post 5-FU administration which was most marked in the small intestine[36]. It has also been suggested the generation of reactive oxygen species (ROS) by NADPH oxidase 1 could also play a vital role at this stage[36]. Nevertheless, similar to Irinotecan, a pro-inflammatory process is initiated by 5-FU-induced intestinal damage and is likely that inflammatory cytokines mediate the subsequent apoptosis noted in intestinal crypts. Pro-inflammatory cytokines such as IL-1β are known to be capable of inducing apoptosis by altering the expression of apoptotic factors such as Bax and Bcl-2[37]. Work by Wu *et al*[38] showed that expression of IL-1 receptor antagonist (IL-1RA), a natural competitive antagonist of IL-1β, was increased in a mouse model of 5-FU-induced intestinal mucositis. Furthermore administration of exogenous IL-1RA resulted in significant reduction in

apoptosis and severity of diarrhea in this murine model; lending support for the role of IL-1β in the pathogenesis of mucositis[39].

A recent study also looked at intestinal mucositis induced by 5-FU in IL-4 knock-out mice. IL-4 is a critical mediator of intestinal inflammation and can function as either a pro- or anti-inflammatory molecule depending on the model of intestinal inflammation. In these mice they reported significantly reduced intestinal damage and inflammation induced by 5-FU after 72 h compared to wild type controls. Furthermore, pro-inflammatory cytokines were increased in wild type controls but not in mice lacking IL-4. The authors conclude that IL-4 has a role in 5-FU induced intestinal mucositis and that removing of IL-4 is effective in preventing pathological alterations secondary to such damage and may improve outcome; supporting the notion that strategy against IL-4 may be a novel logical therapeutic approach for this condition[40].

Keratinocyte growth factor (KGF) was shown to be effective in ameliorating 5-FU-induced intestinal mucositis and prolong crypt stem cell survival in a study by Farrell *et al*[41] but the exact mechanism by which KGF induces its protective effect is as yet not fully understood.

OXALIPLATIN

Oxaliplatin monotherapy seldom results in diarrhea but rather its main dose limiting toxicity results from drug associated neuropathy. As such, several animal models exist for oxaliplatin based toxicity but mainly looking at neurotoxicity, with little data on GI toxicity[42,43]. It is known that GI toxicity is potentiated in combination therapy of oxaliplatin with 5-FU in clinical studies but the exact mechanism behind this observed phenomenon is as yet not fully understood. Few studies have investigated GI mucositis resulting from combined 5-FU and oxaliplatin chemotherapy in the animal models and little data exists for the pathophysiology of mucositis with this combination[44]. Further research into the exact molecular pathways involved in mucositis induced by combination therapy is warranted.

TARGETED THERAPY

Monoclonal antibodies to EGFR such as cetuximab and panitumumab are known to cause diarrhea, though for cetuximab the severity is usually mild[45]. Bevacizumab, a monolconal antibody against VEGF seldom causes diarrhea but is associated with a risk of intestinal perforation, most likely secondary to tissue hypoxia due to inhibition of angiogenesis[46].

However, diarrhea is a well-recognized side effect of oral tyrosine kinase inhibitors. Small molecular targeted chemotherapeutic agents such as regorafenib have been shown to be efficacious in solid tumors and are being increasingly used in the treatment of metastatic colorectal cancer[47]. However it is likely that the mechanism behind their enterotoxicity is different from diarrhea generated by cytotoxic agents. In a rat model of diarrhea induced by lapatinib, an oral tyrosine kinase inhibitor used in the treatment of breast cancer, no significant histopathological changes was noted in the intestinal mucosa despite the development of diarrhea, suggesting an alternative pathway other than the inducement of GI mucositis. Further work to elucidate the exact pathogenesis of this GI specific side effect for this class of agent is warranted and is reportedly underway[48].

SURVIVAL BENEFIT

Chemotherapy is notable for significant toxicities that impact on patient quality of life during therapy and can lead to delay in treatment cycle, dose reduction or drug modification. However in some clinical studies it was noted that modifications to treatment secondary to side effects did not reduce the overall efficacy of the treatment regime[49]. Furthermore the occurrence of certain toxicities could serve as a predictive indicator for improved outcome post treatment. In the treatment of lung cancer with tyrosine kinase inhibitors the development of skin rash is associated with improved response rates[50]. Similarly, diarrhea consequent to sorafenib is a predictor of positive outcome in patients undergoing chemotherapy for advanced hepatocellular carcinoma[51]. With regards to treatment in the setting of CRC; an association between increased incidence of side effects and improved survival is observed. Twelves *et al*[52] demonstrated during post-hoc analysis of the X-ACT trial that the occurrence of hand-foot syndrome (HFS) was associated with better outcome in patients treated with capecitabine. Another study (AIO KRK-0104 trial) looked at the use of capecitabine in combination with other agents including oxaliplatin, irinotecan and cetuximab in the setting of metastatic CRC also found a correlation between skin toxicities triggered by capecitabine and progression-free and overall survival[53]. Hofheinz *et al*[54], 2012 performed a combined analysis of this trial and another rectal cancer trial using the same chemotherapy regimen and concluded that patients with HFS had improved survival compared to those with did not develop this skin toxicity. Interestingly GI toxicity and diarrhea were significantly more common in patients with HFS but not often co-incident with haematological toxicities. The reason for this phenomenon is not yet fully understood but one may speculate that both the mucosal tissue and skin are more susceptible to chemotherapeutic agents that induce apoptosis compared with haematopoiesis. In contrast, the development of skin reaction during cetuximab therapy was shown to be associated with response and survival in metastatic CRC, although no increased GI toxicity was observed in a study by Cunningham *et al*[19] in 2004. This indicates that differential susceptibility of the mucosa to drug-induced toxicities and potential survival benefit may share a common underlying mechanism of action. There is as yet no study to suggest an association between CID and treatment response in chemotherapy for CRC but

this should be evaluated further in clinical studies.

CHEMOTHERAPY EFFECTS ON THE IMMUNE SYSTEM

As the chemotherapeutic agents used to treat cancer cells generate GI toxicity *via* the induction of apoptosis and subsequent inflammation; it is hypothesized that they may also have a beneficial effect on cancer survival by activating an anti-tumor immune response in cancer patients. This concept was supported by findings that cancer cell lines treated *ex-vivo* with certain cancer treatment modalities including chemotherapy can act as a cancer vaccine in animal studies[55,56]. It is now believed that a competent immune system plays a very important role in the efficacy of cancer therapy and that treatment will give the best chance of success when the tumor can be induced to undergo a process of programmed cell death that incites an adaptive immune response, the so called "immunogenic cell death" (ICD)[57]. This process, when activated, leads to the stimulation of T cells by antigen presenting cells such as dendritic cells (DC) through capture, processing and presentation of antigens to naive $CD4^+$ and $CD8^+$ T cells which in turn elicit an anti-tumor response[58].

While apoptosis is generally thought to be immunologically silent, ICD is characterized by the release or exposure of a range of substances called damage-associated molecular patterns (DAMPs), which can trigger an immune response. Of the DAMPs, it appears that the release of extracellular ATP, high mobility group protein B1 (HMGB1) and the exposure of calreticulin (CRT) on the outer membrane of the dying cell are vital for the initiation of ICD[59]. The emission of these DAMPs are triggered by anti cancer drugs and treatments with the ability to induce ICD; known as ICD inducers. These ICD inducers exert their influence in the release of DAMPs through the induction of endoplasmic reticulum (ER) stress in cancer cells and generation of reactive oxygen species (ROS). Both ER stress and ROS work to activate signaling pathways which help to traffic DAMPs to the extracellular space[60]. ICD inducers can be classified into two groups based on the selectivity for the ER in the generation of ER stress. Type 1 ICD inducers act on cytosolic proteins and targets not associated with ER to induce apoptotic cell death which in turn results in ER stress through secondary effects. Examples of type 1 ICD inducers include mitoxantrone, oxaliplatin, cyclophosphamide and γ-irradiation. In contrast, type 2 ICD inducers which include coxsackievirus B3 and hypericin-based photodynamic therapy (PDT) selectively target ER for the generation of ER stress by altering its homeostasis[59].

While the mode of action and the resultant ER stress could be qualitatively different between the ICD inducers, the components of DAMPs are shown to have an immunomodulatory function. Extracellular release of ATP is a strong "find me" signal for monocytes *via* P2Y2 receptors and enhances their recruitment to apoptotic cancer cancers[61]. Exposure of CRT on cell surface of cancer cells undergoing ICD facilitates phagocytosis by DCs which present antigen and activate cytotoxic T-lymphocytes to give an anti-tumor response. Release of extracellular HMGB1 binds to various receptors such as TLR2, TLR4 and receptor for advanced glycosylation end products (RAGE) and in doing so stimulates an inflammatory reaction with the production of pro-inflammatory cytokines which has been found to be vital for the immunogenicity of ICD[61]. Indeed, the interaction between HMGB1 and the TLR-4 receptor on DCs is integral to this process, as a clinical study showed that a polymorphism of TLR-4 that affects the binding of HMGB1 is associated with early relapse of breast cancer[62]. This phenomenon was also observed in metastatic CRC, where Tesniere *et al*[63] showed that patients with normal TLR4 allele have an increased progression-free and overall survival compared with those bearing a loss-of-function TLR4 allele, in a trial involving the use of oxaliplatin-based chemotherapy regime. In addition, they found that this genetic polymorphism did not affect survival in patients with surgically resected CRC who did not undergo adjuvant chemotherapy; highlighting the major role of host immunity and inflammatory responses in determining outcome of chemotherapy in CRC.

CONCLUSION: SUPPORTIVE CARE FOR PATIENTS AND DEVELOPMENT OF NEW DRUGS

With chemotherapeutics in CRC have immunological benefits in addition to their cytotoxic effects, it is imperative that GI side effects are minimized to optimize dosing for treatment so that the best outcome can be achieved. Current management options for CID includes supportive care by symptomatic relief but there is increasing interest in regulating GI mucositis as a means to prevent and treat CID.

LOPERAMIDE

Loperamide is a non-analgesic opioid which helps with diarrhea by decreasing intestinal motility[25]. It is proven to be safe and commonly used in acute and chronic diarrhea in a variety of clinical settings[64]. It is also used as first line management of diarrhea in chemotherapy[65]. In regimens involving irinotecan, high dose loperamide was able to control symptoms to improve tolerability of the drug and to enhance effectiveness of therapy[66]. However its efficacy seems to be limited to mild to moderate diarrhea as a study showed that only 52% of patients who develop grade 3-4 CID responded to loperamide in a CRC cohort undergoing 5-FU-based chemotherapy[67]. Nevertheless its safety profile and affordability make it a worthwhile first line therapy to which other treatment options can be added.

OCTREOTIDE

Octreotide is a somatostatin analogue that has also shown to be effective in managing both secretory and malabsorptive diarrhea in several gastrointestinal disorders including short bowel syndrome and neuroendocrine tumors[68]. Its main mechanism of action is by binding to somatostatin receptors in the GI tract which affect a slow-down in transit time mainly in the small bowel. It also inhibits gut hormones reducing gastric, pancreatic and intestinal secretions, thereby helping to limit excess fluid that is needed to be resorbed by the colonic mucosa[69]. Several clinical studies have shown that the use of octreotide is effective in the treatment of CID[70-73]. There is also evidence that octreotide is more effective than loperamide in 5-FU based regimen[74]. Recent guidelines recommended the use of octreotide at a dose of $\geqslant$ 100 μg subcutaneously twice daily for the control of diarrhea in chemotherapy patients in whom loperamide fail to achieve an adequate response[75].

CELECOXIB

There has been an interest in the theoretical use of celecoxib in CID due to its anti-inflammatory properties, which were thought to ameliorate GI mucositis[76]. In addition, a supposedly anti-tumor effect with COX-2 inhibition makes it attractive as a potential adjunct in the treatment of solid malignancies. These anti-diarrheal and anti-tumor observations were demonstrated in rat models with irinotecan induced diarrhea[76]. However, a phas I study investigating the use of celecoxib in patients undergoing irinotecan based chemotherapy for advanced solid tumors did not show any benefit in CID[77]. Another study by Villalona-Calero *et al*[78], also found that the addition of celecoxib in combination with irinotecan did not improve tolerability of chemotherapy. Further work is needed to define the role of COX-2 inhibition in GI mucositis and its translation to clinical application in the treatment of CRC.

BUDESONIDE

Budesonide is a glucocorticoid with topical anti-inflammatory properties. It has been shown to be effective in the treatment of various inflammatory conditions, including inflammatory bowel diseases[79,80]. It has an extensive first pass metabolism effect in the liver and thus has limited systemic side-effect profile. Its efficacy in GI mucositis and CID was investigated in the clinical setting and an early short report noted improvement in the severity and duration of diarrhea in patients with irinotecan or 5-FU induced CID which was refractory to loperamide therapy[81]. A subsequent randomised placebo controlled trial also noted a reduction in the frequency of diarrhea when budesonide was used as a prophylactic measure but their study did not reach statistical significance. Based on their findings, it was concluded that further trials are warranted[82].

GLUCAGON-LIKE PEPTIDE-1 AND -2

Glucagon-like peptides (GLPs) are peptides which are synthesized and secreted by enteroendocrine L cells located in the GI tract. These molecules are involved in various homeostatic functions in our body, including the regulation of nutrient assimilation and satiety. When stimulated, L cells secrete GLP-1 and GLP-2 in equimolar quantities. Both peptides exert their effect by binding to their receptors, GLP-1 receptor (GLP-1R) and GLP-2 receptor (GLP-2R) respectively. GLP-1R is expressed widely in the body, including in pancreatic tissue, the GI tract, heart, kidney and nervous tissue. In contrast GLP-2R is expressed mainly in the GI tract and CNS. The differential distribution of their receptors partly explains the distinct physiological effects of GLP-1 and GLP-2; with GLP-1 exerting an influence in glucose homeostasis as an incretin hormone while GLP-2 has no significant incretin effects. Instead, GLP-2 has been noted to have potent intestinal trophic effect, promoting crypt cell proliferation and villous growth of the jejunum and ileum[83]. In addition, GLP-2 enhances intestinal barrier function and has a cytoprotective effect on intestinal mucosa[84]. Exogenous GLP-2 has been shown to be protective against various intestinal insults, including ischemia-reperfusion-induced and irradiation induced injury[85,86]. In animal models of inflammatory bowel disease, the administration of GLP-2 was shown to have significant anti-inflammatory effects, and ameliorated weight loss associated with ileal and colonic inflammation[87]. There is therefore an intense interest in the ability of GLP-2 to reduce inflammation in GI mucositis and CID. In a murine model of CID, Boushey *et al*[88] demonstrated that a GLP-2 analogue was able to enhance survival and reduce weight loss while having little effect in chemotherapy effectiveness on the tumor. Furthermore they observed that this effect was driven in part by anti-apoptotic effects on intestinal cells expressing GLP-2R. Yamazaki *et al*[89], 2004 showed that increasing GLP-2 levels by pharmacological means significantly attenuated intestinal damage measured by reduction of small intestinal wet weight in 5-FU treated mice. Other studies also noted similar changes and a reduction in inflammatory cells suggesting an immunomodulatory effect of GLP-2 in CID[90,91]. Intriguingly GLP-1 has also been found to have an intestinal trophic effect and treatment with GLP-1 ameliorated GI mucositis induced by 5-FU in mice[92]. Clinical studies are therefore warranted to translate such encouraging pre-clinical data to the treatment of CID *via* the GLP pathway.

CONCLUSION

GI toxicity from systemic chemotherapy in CRC remains a significant burden to patients limiting quality of life and impacting on optimal dosing for effective treatment. Recent advances highlight the importance of inflammation in the pathophysiology of GI mucositis and also bring to the attention its potential role for enhanced cancer survival post chemotherapy by triggering immunogenic cell

death. Strategies to nullify the undesirable yet common side effects of GI toxicity by addressing inflammatory changes triggered during mucositis are currently in development; with agents targeting the GLP pathway showing great promise in pre clinical studies. However, it is important to note that any such agents developed should not interfere with the efficacy of chemotherapy treatment and the complex interplay between side effects of inflammation and inflammation driven immunogenicity will need to be considered.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Personalized surgical management of colorectal cancer in elderly population

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Abstract

Colorectal cancer (CRC) in the elderly is extremely common but only a few clinicians are familiar with the complexity of issues which present in the geriatric population. In this phase of the life cycle, treatment is frequently suboptimal. Despite the fact that, nowadays, older people tend to be healthier than in previous generations, surgical undertreatment is frequently encountered. On the other hand, surgical overtreatment in the vulnerable or frail patient can lead to unacceptable postoperative outcomes with high mortality or persistent disability. Unfortunately, due to the geriatric patient being traditionally excluded from randomized controlled trials for a variety of factors (heterogeneity, frailty, *etc.*), there is a dearth of evidence-based clinical guidelines for the management of these patients. The objective of this review was to summarize the most relevant clinical studies available in order to assist clinicians in the management of CRC in the elderly. More than in any other patient group, both surgical and non-surgical management strategies should be carefully individualized in the elderly population affected by CRC. Although cure and sphincter preservation are the primary goals, many other variables need to be taken into account, such as maintenance of cognitive status, independence, life expectancy and quality of life.

Key words: Elderly; Colorectal cancer; Surgery; Personalized treatment; Geriatric assessment

Core tip: More than 50% of colorectal cancer cases are diagnosed in patients over 70 years of age. As the geriatric patient is traditionally excluded from randomized controlled trials for a variety of factors (heterogeneity, comorbidities, polypharmacy, inability to consent, *etc.*) there is a dearth of evidence-based clinical guidelines for the management of these patients. Although cure and sphincter preservation are the primary goals, many other variables need to be taken into account, such as the maintenance of cognitive status, independence, life expectancy, and quality of life. Personalized and patient-centered care should be the goal when caring for elderly patients with colorectal cancer.

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INTRODUCTION

The world population is aging[1]. This process is especially evident in Western society due to a combination of increased life expectancy and a reduced birth rate[2]. With

aging, the incidence and prevalence of cancer increases[3,4]. With a median age of 70 years at diagnosis and an incidence drastically increasing with age, colorectal cancer (CRC) is by far one of the most commonly diagnosed malignancies in the elderly. It has recently been demonstrated that the highest risk of being diagnosed with CRC is between 80 and 89 years of age; thus, in future decades, CRC will constitute a major burden for health care systems[5]. Surgical resection is still the cornerstone of curative treatment for this disease. Although improvements in perioperative care, surgical techniques and the introduction of multimodal treatment have made surgery feasible for the vast majority of patients, elderly cancer patients still represent a challenge for the surgeon[6].

The intrinsic reduction in tolerance to stressors, and the frequent presence of one or more disorders in addition to the cancer increase the risk of a poor surgical outcome in elderly patients undergoing cancer-related surgery. It is important to remember that there is great variation in individual health status with increasing age; as a consequence, a multidimensional approach by a multidisciplinary team should be incorporated into daily practice before planning treatment[7].

For this population group, the goal of each assessment is to customize optimal management according to physiological/biological age instead of crude chronological age in order to avoid overtreatment of the frail and undertreatment of fit senior adults. Considering the frequent complexity of geriatric patients and their underrepresentation in randomized studies[8], surgical decisions made on an individual basis are increasingly more important. Surgical treatment is a potential promoter of permanent disability in elderly patients, but this is mainly the case for vulnerable and frail individuals[9]. We therefore reviewed the key elements of personalized surgical management for CRC in the elderly population (Table 1).

PREOPERATIVE CONSIDERATIONS BEFORE CRC SURGERY IN THE ELDERLY

Elderly patients are a heterogeneous population, often presenting with various degrees of coexisting medical and psychosocial issues which need to be weighed before selecting and initiating surgical treatment. Therefore, the importance of a holistic evaluation, a multidisciplinary approach and careful preoperative screening are emphasized as first steps in providing a more tailored approach to ensure the best treatment among different therapeutic strategies. Furthermore, specific considerations regarding the perspectives and expectations of elderly patients regarding CRC surgery are addressed and attention is also focused on prehabilitation, a promising aspect in the onco-geriatric field.

Multidisciplinary approach

One of the greatest challenges of modern medicine is the promotion of close collaboration among the specialists involved in the different aspects of a patient's management, favoring a patient-oriented approach. This is particularly true within the field of geriatric oncology where the mixture of a disease- and a patient-oriented approach seems to be the most appropriate modality for better treatment of this complex and heterogeneous population. By close interaction, achieved by the creation of multidisciplinary teams, physicians must assess patient malignancy as well as their global health status, including comorbidities, treatment, psychosocial issues, nutritional and functional status.

Close collaboration among specialists has already been attempted in heterogeneous settings; clinical studies have shown the benefits of interdisciplinary team care in both inpatient (*e.g.*, acute care, elective orthopedic surgery)[10,11] and outpatient management (*e.g.*, fall prevention, functional recovery)[12,13]. Recent attempts involving a growing interest in collaboration between cancer centers and geriatric departments as regards geriatric oncology have been described[14]. A multidisciplinary approach, where surgeons work side by side with anesthesiologists, geriatricians, physiotherapists, nutritionists and other ancillary professionals, can provide favorable surgical outcomes (*e.g.*, disability-free life expectancy and overall survival)[15] through improved selection of candidates for intervention and a more considered exclusion of patients characterized by high risk profiles or a poor prognosis.

Holistic evaluation

In this section, the most important aspects which have a notable impact on the morbidity and mortality rates associated with CRC surgery are discussed in an attempt to obtain an accurate presurgical evaluation. Recently, checklists for the optimal preoperative assessment of the geriatric surgical patient have also been made available in order to pursue an optimal preoperative assessment[16]. Sarcopenia, with a prevalence ranging from 11% to 50% in the population 80 years of age or older, is often related to the aging process and is recognized to be associated with decreased survival in cancer patients and with an elevated risk of poor outcome in CRC patients undergoing surgical resection[17,18]. A recent study by Lieffers *et al*[19] showed that, in CRC patients 65 years of age and older, sarcopenia was independently predictive of postoperative infections (OR = 4.6; 95%CI: 1.5-13.9), convalescent care (OR = 3.1; 95%CI: 1.04-9.4), and significantly associated with a prolonged length of hospital stay (15.7 ± 9.8 d *vs* 11.8 ± 6.4 d for non-sarcopenic patients).

Impaired nutritional status is a common finding among elderly patients, especially among those admitted to hospital[20]. It is estimated that 40% of elderly hospitalized patients with cancer are at risk of malnutrition, which has been found to be associated with prolonged hospital stays, and increased morbidity and mortality in patients undergoing elective gastrointestinal surgery. Sungurtekin *et al*[21] preoperatively assessed the nutritional status in 100 patients undergoing major abdominal surgery using different assessment tools and found that malnourished patients were at a higher risk of complications, with

Table 1 Key elements of personalized surgical management for colorectal cancer in the elderly population

Preoperative considerations before CRC surgery in the elderly
Multidisciplinary approach
Holistic evaluation
Preoperative risk screening tools in surgery
Prehabilitation
Perspectives and expectations regarding CRC surgery
Personalized surgical management of colon cancer in the elderly
Stage Ⅰ-Ⅲ colon cancer
Stage Ⅳ colon cancer
Malignant bowel obstruction in the elderly
Laparoscopic approach for colon cancer in the elderly
Personalized surgical management of rectal cancer in the elderly
Specific considerations regarding morbidity and mortality
Functional results
Laparotomy *vs* laparoscopy for TME
The Habr-Gama effect
Postoperative recovery after CRC surgery in the elderly
Laparoscopic approach and independence
Rapid rehabilitation program
Considerations regarding QoL

CRC: Colorectal cancer; TME: Total mesorectal excision; QoL: Quality of life.

the ORs for the association between malnutrition and complications varying from 1.92 to 9.85 depending on the assessment tool used. Furthermore, higher death rates were found in the malnourished group. Similar findings were observed among gastrointestinal cancer patients[22]. Regarding CRC patients, Mohri *et al*[23] found that malnutrition was an independent predictor of poor survival (OR = 2.04; 95%CI: 1.39-3.09) and was significantly correlated with the incidence of postoperative complications, especially serious ones, in a cohort of 365 patients (171 patients > 65 years old).

With a median of 4 comorbidities present at the time of CRC diagnosis, multimorbidity, defined as the occurrence of multiple diseases in the same individual, often affects older patients with CRC. Available evidence clearly indicates that comorbidities are one of the major predictors of surgical morbidity, mortality and survival. Regarding survival, a retrospective study of a cohort of 29733 patients 67 years of age or older with a primary diagnosis of stage Ⅰ-Ⅲ CRC showed that comorbidities exert a substantial influence on survival as the predicted 5-year survival in patients with stage Ⅰ CRC and comorbidities was approximately 50% *vs* 78% for patients with stage Ⅰ cancer without comorbidities[24]. Zingmond *et al*[25] found that, of 56621 CRC patients undergoing tumor resection, those with a higher Charlson comorbidity index (CCI) were significantly associated with postoperative complications. Similarly, Tan *et al*[26] showed that the CCI was an independent predictor of morbidity in a population of 121 octogenarians undergoing CRC surgery. Similarly, Ouellette *et al*[27] demonstrated that CCI was associated with a longer length of stay, perioperative mortality, and overall mortality in 239 CRC patients.

Disability is a crucial predictor of a poor postoperative outcome. A recent study identified any functional dependence to be the strongest predictor of 6-mo mortality in 110 elderly subjects (mean age, 74 ± 6 years) undergoing major surgery requiring postoperative intensive care unit admission[28]. Cancer patients defined as being functionally dependent according to the validated instrumental activity of daily living were found to have a 2- to 3-fold increased risk of postoperative morbidity compared with those defined as independent[29,30]. Although for the most part, attention has to be drawn towards the assessment of comorbidities, nutritional impairment and disability, physicians should not forget to focus on elderly psychosocial issues as their presence has been associated with an increased risk of mortality and poor surgical outcome[31]. Hu *et al*[32] have recently examined the role of dementia on surgical outcome in 207693 patients 60 years of age or older who underwent inpatient major surgery. The authors showed that patients with dementia had a significantly higher overall postoperative complication rate compared with controls (adjusted OR = 1.79; 95%CI: 1.72-1.86). Finally, despite the fact that the role of depression on surgical outcome in cancer patients undergoing tumor-related surgery needs additional future clarification, presurgical depression has been found to be an important independent contributor to medical morbidity in patients undergoing cardiac surgery[33].

Preoperative risk screening tools in surgery

The ideal objective of the preoperative assessment of elderly cancer patients should be the correct definition of those who are characterized by frailty, a multifactorial and continuous decline of multiple physiologic systems which still represents a challenge to the surgical community. Studies focusing on older patients undergoing elective cardiac and non-cardiac surgery estimate the prevalence rates of frailty to vary from 41.8% to 50.3%[9]. Over time, several comprehensive assessment scales have been developed to pursue this goal and to enable risk stratification in cohorts of elderly people. Comprehensive geriatric assessment (CGA), a multidisciplinary diagnostic process which evaluates multiple aspects of the elderly has been broadly used for this purpose within the geriatric oncology setting. There is evidence that abnormalities in preoperative geriatric assessment are strongly related to the occurrence of adverse postoperative outcomes including institutionalization, prolonged length of hospitalization, morbidity and mortality[30,31,34,35].

Furthermore, a review focusing on frailty in the elderly surgical patient states that frailty is predictive of mortality, postoperative complications and institutional discharge in elderly patients undergoing both cardiac and non-cardiac surgery[36]. Regarding CRC surgery, a recent study has found that CGA was able to predict surgical morbidity in a cohort of 178 elderly CRC patients where the CGA-defined group of frail individuals was found to be significantly associated with severe complications (OR = 3.13; 95%CI: 1.65-5.92)[37]. Patients were defined as frail when fulfilling one or more of the following criteria: personal activity of daily living score less than 19, any

grade 4 comorbidity according to the cumulative illness rating scale (or more than 2 grade 3 comorbidities), more than 7 daily medications, a Mini Nutritional Assessment score of less than 17, a poor score on the mini mental state examination (< 24) and on the geriatric depression scale (> 13).

However, two issues need to be carefully pointed out when focusing on frailty. First, despite years of research, the quantification of frailty remains a controversial and complex topic[38] and second, the use of preoperative geriatric assessment hardly seems applicable in daily practice as it is time consuming. Thus, rapid tools have been developed with the aim of quickly identifying frail patients[30,39-41]. Among the above-mentioned tools, the timed up and go (TUG) test, a test used to assess a person's gait speed and mobility, seems to be the most promising[42]. Poor performance on this test correlates with the presence of other aspects of frailty; thus, its use as a rapid and simple means of stratifying preoperative risk in the elderly seems reasonable. Since its introduction, several studies have pointed out a clear correlation between a prolonged TUG and poor functional status, cognitive impairment and fall risk[43]. Furthermore, a slower TUG test has recently been demonstrated to predict postoperative complications, 30-d readmission, institutionalization and 1-year mortality in a cohort of 272 elderly patients undergoing elective surgery[28]. Clarifying the role of TUG and other forms of rapid presurgical assessment in the specific field of geriatric oncology is imperative. The international prospective project preoperative risk estimation for onco-geriatric patients (PREOP study) has recently been launched with the aim of providing new evidence regarding the predictive value of these new tools and comparing them with more complex forms of geriatric assessment[44].

Prehabilitation

Despite modern and sophisticated efforts for decreasing postoperative morbidity and mortality, and facilitating full recovery after CRC surgery, there is evidence that, 6-9 wk after major abdominal surgery, many patients are not back to their active lives[45,46]. Prehabilitation is a modern strategy, gathering together all the initiatives carried out from the time of diagnosis to the time treatment starts in order to improve functional capacity and functional recovery. Cancer prehabilitation is a novel topic compared with the amount of knowledge of post-treatment rehabilitation programs and outcomes for both cancer and non-cancer patients[47].

Interestingly, the first study on prehabilitation was published in 1946, describing nutritional and physical training, and even recreational intervention in order to turn the unfit military into robust soldiers ready for the battlefield[48]. In recent years, cancer patient prehabilitation has become more and more intriguing for surgical oncologists as a result of the great benefits shown in the fields of orthopedic and cardiac surgery, even for the elderly[49-56]. Medical prehabilitation clearly includes the management and optimization of preoperative conditions, such as diabetes, cardiovascular function and the promotion of smoking cessation. Moreover, the goal of this strategy should not only focus on muscle strength reinforcement but also on the nutritional and emotional/psychological management of patients undergoing major surgery for cancer. The work that Carli *et al*[57] have accomplished in recent years has been of great value in the daily life of clinicians and patients. They actually showed that functional capacity regarding CRC surgery was improved by prehabilitation, whether by adherence to a strenuous preoperative activity schedule (bike and muscle strengthening exercises) or by a 30-min walking and breathing exercise regimen 3 times a week[57].

On the other hand, many questions are still open as to how older adults undergoing cancer surgery may or may not benefit from perioperative regimens[58]. The Enhanced Recovery After Surgery (ERAS) guidelines for patients undergoing colorectal surgery have recently clearly defined any potential benefits from a pretreatment regimen as inconsistent[59]. The ERAS panel basically pointed out the lack of large randomized trials in the CRC field, the low adherence of patients to the prehabilitation regimen and the need for a prolonged time period from diagnosis to surgery (at least 4-6 wk) in order to observe tangible improvement in postoperative outcomes. The majority of confusion regarding the potential usefulness of this intervention comes from incorrect expectations regarding prehabilitation outcomes. What is clear is that prehabilitation is not a substitute for good surgical and tailored postoperative treatment, above all in the elderly. As a consequence, it does not reduce the morbidity and mortality rate. Prehabilitation improves functional recovery and perhaps patient independence and active life expectancy time.

Li *et al*[60] recently showed how a trimodal prehabilitation program dramatically changed postoperative functional walking capacity, self-reported physical activity and health-related quality of life (QoL). The randomized trial was designed for CRC patients awaiting surgical treatment and included 30 min of walking and breathing exercises 3 times a week, a nutritional supplement of up to 1.2 g/kg body weight and anxiety reduction techniques. The mean age of the 42 patients enrolled and the 45 patients in the control group was 67.4 ± 11 years; a prehabilitation protocol was carried out for a mean time of 33 d (range, 21-46 d). Interestingly, the patients in the intervention group increased the distance covered at the 6-min walking test during prehabilitation, surpassing the preoperative results of the control group. Four and 8 wk after surgery while control patients' physical ability declined and did not reach their pretreatment level, rehabilitate patients regained the ability to walk farther than their preoperative baseline. The same trajectory was shown for self-reported physical activity while anxiety and depression were shown to be way below the patient baseline 4 wk postoperatively. Even more interestingly, fewer postoperative complications were recorded in pa-

tients who improved their walking ability during prehabilitation while people whose functional capacity declined during the pretreatment time had poorer outcomes. This might help in considering the response to the prehabilitation regimen to be an additional screening tool for elderly patients undergoing surgery for cancer.

Several issues regarding the feasibility and effectiveness of this approach have still not been completely resolved. The lack of time which often forces surgeons to bring elderly patients with CRC to the operating room sooner rather than later because of impending obstruction or perforation might reduce the practicability for a very large number of patients. At the same time, lack of adherence to prehabilitation regimens is indeed higher in the elderly, above all in cases of inconsistent family or financial support. On the other hand, the results obtained before CRC surgery are so promising for restoring active life and independence in this frail group of patients that it may be worth a try, above all, for those patients who are able to wait 4-6 wk before surgery (*e.g.*, neoadjuvant therapy). Good clinical data and larger trials focused on elderly patients are needed to eventually shed light on this fascinating field.

Perspectives and expectations regarding CRC surgery

Patient perspective is essential in establishing a proper understanding of the QoL goals and achieving good postoperative outcomes for senior adults with CRC. Despite the prevalence of CRC in the elderly population and the increasing requirement for QoL measurement, not many studies have been published which focus on patient experience regarding their cancer treatment[61]. In recent years, some qualitative information has been gained from studies designed for younger patients where "uncertainty", "fears for cancer recurrence", "pain", "fatigue", "managing on a day to day basis" and "feeling alone" were described as the highest concerns of CRC patients[62-64]. Mental and physical health seemed to be interrelated in both young and senior adults with cancer as reported by Weaver *et al*[65], affecting their perspective regarding their disease and the expectations as to the cure they were undergoing.

In an interesting review, Banks *et al*[66] were able to analyze self-reported questionnaire-based data from 89574 Australian men and women with cancer sampled from the Medicare database. In their study, they were able to conclude that, although approximately 8% of people suffer from severe psychological distress, "the risk of psychological distress in individuals with cancer relates much more strongly to their level of disability than it does to the cancer diagnosis itself". Disability and lack of independence in the activities of daily living seem to impact cancer patients more than the cancer prognosis *per se*. Unfortunately, the cohort of patients analyzed also included non-CRC patients 45 years of age and older but, despite this, it seemed quite feasible to translate the results to our study population.

Among the possible stressors, having a stoma has been historically considered as a factor which increases psychological distress in patients with CRC. This fact has also been reconsidered in the past few years. A large meta-analysis on the impact of a stoma forming procedure [abdominal perineal resection (APR) *vs* low anterior resection (LAR)] on 1443 patients with CRC failed to show a reduction in the QoL of patients with fecal diversion. The mean age in the two groups was 66.3 ± 6 and 65.6 ± 6 years for APR and LAR, respectively[67]. This important finding was again confirmed by a smaller but more recent study from the Netherlands where no difference was seen in terms of health-related QoL, emotional function and understanding of the illness among elderly rectal cancer patients with or without a stoma[68]. This may indicate that having a stoma and the risk of incontinence are considered equally troublesome for patients. Regardless of the large amount of literature on the preoperative assessment of onco-geriatric patients, not many studies have been carried out which focus on elderly patients' needs and expectations before and after CRC surgery.

Patient-centered outcome studies should be implemented in the onco-geriatric field in order to face modern health care system challenges[69]. Data seem to suggest that disability and lack of independence are considered more important than the cancer diagnosis *per se*. The risk of postoperative disability, and not just the risk of having a fecal diversion, need to be fully discussed with patients and family with the goal of promoting faster functional recovery and regaining independence.

PERSONALIZED SURGICAL MANAGEMENT OF COLON CANCER IN THE ELDERLY

Stage Ⅰ-Ⅲ colon cancer

Surgery represents the treatment of choice for stage I to stage Ⅲ colon cancer[70]. Given that many advances have been achieved in surgical techniques, anesthesia and perioperative supportive care, it is now accepted that age *per se* is not a contraindication for surgery in senior colon cancer patients[71], even if it is still hard to overcome the general thinking that a less aggressive and radical approach should be provided for this population[72,73]. A recent study by Dekker *et al*[74] described a population-based analysis of 9397 stage Ⅰ-Ⅲ CRC patients operated on in the Netherlands from 1991 to 2005. They showed that decreased survival in the elderly is mainly due to differences in early mortality. Elderly CRC patients who survived the first year had the same cancer-related survival as younger patients; therefore, treatment of elderly CRC patients should focus on perioperative care and the first postoperative year.

It is well known that elderly patients have an increased number of comorbidities which leads to a higher rate of morbidity and mortality[75]. A systematic review including 34194 patients conducted by the Colorectal Cancer Collaborative Group[76] compared the outcomes of patients

65-74 years of age, 75-84 years of age and those 85 years of age and over with those 65 years of age or younger. The study showed that elderly patients had an increased rate of comorbidities, they were more prone to undergo emergency surgery and they were less likely to undergo curative treatment. Surprisingly, the same review demonstrated that, even if overall survival was reduced, cancer-specific survival was not. The two studies definitely showed that, when carefully selected, even very elderly patients benefit from surgery since a large proportion survive for 2 or more years after surgery[7]. A study conducted by the Colon/Rectum Cancer Working Group recruited a total of 19080 CRC patients (2932 over 80 years of age) to analyze the impact of the risk factor "age" on early postoperative results. The rate of surgically-specific postoperative complications was identical among younger and elderly patients. Also in this case, elevated morbidity and mortality rates were found to be associated with increasing age due to more cardiovascular and pulmonary adverse events[77]. Kunitake *et al*[78] described outcomes of 83987 elderly colon cancer patients identified in the California Cancer Registry. Octogenarians and nonagenarians had worse outcomes in terms of morbidity, mortality and readmission rates compared with younger patients. An increased number of comorbidities and emergency procedures were found to be consistent risk factors for adverse outcomes while, interestingly, adjuvant chemotherapy and surgery in high volume hospitals were associated with lower odds of in-hospital and 1-year mortality.

Furthermore, a pooled analysis conducted by Sargent *et al*[79] provided good evidence to support the fact that 5-fluorouracil adjuvant therapy is well tolerated by elderly patients with benefits comparable to younger patients in terms of overall survival. On the other hand, no benefits from the addition of newer agents (*e.g.*, irinotecan and oxaliplatin) have been shown in large multicenter trials[80]. Since a correlation with poorer outcomes[81] is well known, emergency procedures should be avoided whenever possible, always considering bridge solutions to improve performance status.

Stage IV colon cancer

Twenty to 34% of patients with CRC present with synchronous liver metastases, and a higher rate will develop after primary diagnosis. The role of surgery in advanced CRC is limited. Guidelines from the National Comprehensive Cancer Network recommend that patients with stage IV CRC should undergo surgery only if they are symptomatic (*e.g.*, bleeding, obstruction, perforation) or have a potentially resectable metastatic localization. Despite progress in the quality of chemotherapeutic agents, liver resection still remains the only chance for long-term survival in patients with CRC liver metastases.

In recent years, several studies have evaluated the feasibility of liver resection for colorectal metastases. De Liguori Carino *et al*[82] analyzed data from 181 liver resections performed on 178 consecutive senior adult patients. The overall survival rate at 5 years was 31.5%. Similar results were reported by Nagano *et al*[83] who reported 34.1% 5-year survival in 202 elderly patients undergoing surgery for CRC liver metastatic disease. An interesting study evaluated the outcome of liver surgery for colorectal metastases in patients over 70 years of age in a large international multicenter cohort[84]. The elderly were compared to a younger population, and a higher rate of 60-d postoperative mortality and morbidity was found but, surprisingly, the 3-year survival rate was similar in the two groups (57.1% *vs* 60.2% for elderly and younger patients, respectively). Liver resection for CRC metastases in elderly patients can achieve a reasonable survival rate. There should be no upper age limit, but the surgical approach should be planned taking into consideration disease stage, patient life expectancy, performance status and the presence of comorbidities. Benefits related to neo-adjuvant treatment for initially unresectable metastatic disease are still not clear in the younger population; additional data are needed to evaluate possible implications for elderly cancer patients.

Malignant bowel obstruction in the elderly

Bowel obstruction is a frequent presentation of advanced disease, especially in the elderly population[85]. Right colon cancer only rarely presents with obstructing symptoms and, in those cases, surgical treatment is almost always needed. In contrast, left colon cancer is more frequently responsible for bowel obstruction at presentation and its management has been the subject of debate. Several studies have been undertaken to evaluate non-surgical strategies in malignant left-sided large bowel obstruction. Self-expanding metal stents (SEMS) have been proposed since 1991 as a bridge solution to relieve acute symptoms, improve clinical conditions and allow patients to receive elective surgical procedures and to possibly avoid a stoma. Conflicting data are available on the topic. On one hand, some retrospective analyses have suggested that the use of SEMS in the elderly population is an effective and safe therapeutic option compared with primary emergency surgery[86] for both elderly and younger patients[87]. On the other hand, two randomized trials tried to establish whether colonic stenting improved patient outcomes compared with emergency surgery, but neither managed to define a decisive clinical advantage[88,89]. The randomized controlled trial conducted by Cheung *et al*[90] compared a multimodal approach (SEMS positioning followed by early laparoscopic resection) to emergency procedures. The authors concluded that the "endolaparoscopic" approach makes a single stage operation more feasible as it is associated with reduced necessity of a stoma.

Another prospective, randomized controlled trial concluded that SEMS as a bridge to elective surgery (performed after 5-7 d) is associated with lower morbidity, a shorter hospital stay, and equally good long-term survival[91]. Despite this evidence, a recent Cochrane review concluded that the use of colonic stents in malignant CRC obstruction seems to have no advantage in terms of

early mortality and morbidity rates compared with emergency surgery[92]. Additional randomized trials focusing on large sample sizes are needed to achieve clearer evidence regarding the role of SEMS in the elderly population.

Laparoscopic approach for colon cancer in the elderly

In order to face the frequent poor performance status and the elevated number of comorbidities characterizing the elderly CRC population, surgeons have investigated a wide range of possible solutions for improving outcomes. In the last 15 years, several peer-reviewed studies have been published evaluating the feasibility, safety and advantages of the laparoscopic approach for colonic cancer in elderly patients. The vast majority have illustrated that, in the elderly population, minimally invasive surgery reduces overall mortality and morbidity when compared to a laparotomy, and correlates with a shorter hospital stay and faster functional recovery. Furthermore, it has been clearly demonstrated that postoperative outcomes in the elderly did not significantly differ from those of younger CRC patients.

Many studies focusing on postoperative mortality have been published pointing out favorable short-term results, but the majority of them are characterized by a vast heterogeneity in terms of colorectal pathologies including inflammatory bowel diseases, diverticular disease and functional diseases[93,94]. Some of them emphasized similar or even lower short- and long-term mortality rates among patients undergoing elective laparoscopic surgery compared with those undergoing a laparotomy[95,96]. Interestingly, a 10-year retrospective study conducted by Cheung *et al*[97] analyzed long-term survival with a median follow-up of 24 mo in a population of 101 octogenarians who underwent elective laparoscopic surgery for CRC. The overall 5-year survival rate was 51%, slightly less than other reports referring to the general population, but still a noteworthy result. It should also be noted that, in the same study, more than half of the deaths were caused by non-cancer-related conditions, such as coexisting cardiopulmonary diseases.

It is common knowledge that laparoscopic colectomies performed in the neoplastic elderly population are associated with higher rates of complications[98]. Data retrieved from a large prospective, observational multicenter study conducted by the Laparoscopic Colorectal Surgery Study Group including 4823 CRC patients (909 treated laparoscopically) showed that intraoperative and postoperative complications were equally distributed among cancer patients over 75 years of age and younger patients[99]. In particular, no differences were observed regarding anastomotic leaks and the re-operation rate. Cardiac and pulmonary events are the most frequent non-surgical complications and they are often caused by a presurgical coexisting morbidity (*e.g.*, chronic heart failure, atrial fibrillation, chronic obstructive pulmonary disease). A paper presented by Law *et al*[100] found that cardiopulmonary complications were markedly fewer in patients who underwent laparoscopic surgery. This trend was even clearer in patients with concurrent preoperative cardiopulmonary pathological conditions.

A remarkable study conducted by Senagore *et al*[101] illustrated that cardiac and pulmonary postoperative complications were higher in patients 70 years of age or older who underwent open colorectal surgery compared with those who underwent laparoscopic procedures. Moreover, the same study showed that, among those who underwent laparoscopic surgery, the observed morbidity rate was much lower than that predicted by the Physiological and Operative Severity Score for the enumeration of Morbidity and Mortality; the same results were not achieved within the open surgery group, confirming the observed general trend of lower unexpected events with a minimally invasive approach. As previously mentioned, short-term postoperative death in elderly patients is principally caused by non-surgical complications. Thus, reducing this risk will inevitably produce better outcomes. Laparoscopy seems to be markedly effective in achieving this result as the systemic stress induced by the minimally invasive technique appears to be better tolerated[102]. Data regarding intraoperative blood loss and functional recovery are extremely explanatory in confirming this issue; several studies found less bleeding and faster recovery in elderly patients undergoing laparoscopic resections[103-106]. Moreover, this minimally invasive approach has been demonstrated to have better results in terms of postoperative pain, allowing physicians to decrease the use of narcotics and opioids, resulting in a decreased risk of postoperative delirium and, consequently, in shorter hospital stays. Nowadays, laparoscopic colonic resections should be mandatory in the elderly neoplastic population due to the massive evidence of advantages related to this approach[107]. Laparoscopy allows onco-geriatric surgeons to drastically decrease the rate of postoperative complications related to surgery and comorbidities, giving the patients a better chance of fast recovery and long-term survival. There is no longer any need for concern when offering a minimally invasive approach to the elderly population.

PERSONALIZED SURGICAL MANAGEMENT OF RECTAL CANCER IN THE ELDERLY

The management of elderly patients with rectal cancer is frequently influenced by many factors which lead to undertreatment with consequent poorer outcomes as demonstrated in a study performed by Chang *et al*[108] in a group of 21390 patients identified in the Surveillance, Epidemiology, and End Results database (1991-2002). The authors found a decreased use of multimodal treatment, an increased use of local excision and a decreased use of radical surgery. The study also showed that the rectal cancer-specific survival rates decreased as patient age increased. Many surgical and non-surgical options are available for rectal cancer patients. Careful pretreatment

assessment in order to identify fit, vulnerable and frail patients should be routinely incorporated into daily practice, especially in this subgroup of elderly patients with rectal cancer. The main goal is to avoid undertreatment in the fit and to plan personalized management for vulnerable/frail patients.

Specific considerations regarding morbidity and mortality

Despite the fact that individuals over 75 years of age comprise 8%-10% of the overall population, 35%-45% of patients with rectal cancer fall into this subgroup of patients, with an incidence of approximately 135 new cases per 100000 people in the group from 80 to 85 years of age[109-111]. Surgery is still the cornerstone for the treatment of these patients. Regardless of the increased risk of postoperative complications, 5-year cancer-specific mortality is comparable to that of younger patients, emphasizing the similarity of the intrinsic prognosis of the disease[112-114]. Two interesting multicenter studies have confirmed that the increase in postoperative morbidity and mortality (from 0.5% in patients under 50 years of age to 13% in patients over 80 years of age) is not related to age *per se*[115,116]. As expected, according to the American Society of Anesthesiology (ASA) score, emergency surgery, low rectal cancer and advanced tumor stage were responsible for the higher number of postoperative complications. Unfortunately, elderly people with advanced cancer and in a setting of several comorbidities are more prone to undergo emergency surgery. This amount of evidence reinforces the idea that age is not an indication of a poor prognosis but that biological age (also interpreted as diminished functional capacity) is.

Since low rectal cancer is related to an increased risk of complications, interest has been drawn towards understanding the impact of age on postoperative complications. Two studies by Rutten *et al*[109,117], analyzing postoperative complications in elderly patients from a Dutch trial, pointed out an unusual finding: anastomotic leak risk was about 10% in people over 75 years of age and 12% in younger patients ($P = 0.63$) but, after 6 mo, more than half of the elderly patients (57.1% *vs* 8.2%) who experienced an anastomotic complication died. Six-month mortality was 22.9% overall *vs* 7.0%, (relative risk: 3.27; 95%CI: 2.05-5.21) among elderly patients who had a postoperative complication (*e.g.*, sepsis, abscess, cardiac and pulmonary complications) compared with younger patients. Once more, this finding demonstrates that postoperative complications are not tolerated very well by elderly patients, therefore, pointing out the importance of accurately monitoring the postoperative course in this patient population.

Functional results

Rectal cancer surgery has two main endpoints: locoregional control and functional results including sphincter, urinary and sexual functions. A clear and realistic description of the possible consequences of the surgical procedure should be explained to patients and caregivers before planning treatment. Several studies have added data regarding functional results after sphincter-saving surgery in the elderly. Dehni *et al*[118] examined the long-term functional results of a small group of elderly patients compared with young people in whom LAR and coloanal J-pouch anastomosis were carried out. The elderly patients reported more constipation and use of laxatives or enemas but the difference with the younger counterparts was not statistically significant. Furthermore, 91% of patients over 75 years of age were satisfied with their functional results. Both Phillips *et al*[119] and Hida *et al*[120] found that elderly patients experienced the same or even more satisfaction in their bowel habits and sphincter function compared with younger patients.

More interestingly, Ito *et al*[121] prospectively explored the risk factors for fecal incontinence (the Wexner score was used) on 96 patients with poor anal function after restorative rectal surgery. Surprisingly, in univariate analysis, age did not correlate with poor sphincter function while only the extent of the sphincter excision and preoperative chemoradiation therapy did. Impressive data are also available regarding the tendency of a diverting ostomy takedown after LAR in the elderly population. The Dutch trial, including 924 patients who underwent LAR, showed that, of the 616 patients on whom an ostomy was performed during surgery, 19% still had a bowel diversion after 7.1 years of follow-up, and that age was a significant risk factor associated with the decreased likelihood of having their stoma reversed[122]. Advanced age and comorbidities were again significant risk factors for not having a loop ileostomy reversed in a cohort of 964 patients analyzed by David *et al*[123] where 233 (24.9%) patients still had an ileostomy bag after a 3-year minimum follow-up. All these data should increase the evidence that age is not a contraindication for radical restorative rectal surgery but that the frailty and functional capacity of individual patients should be weighed when major surgery for rectal cancer is planned in this cohort of patients[124]. When neoadjuvant treatment is considered for rectal cancer, clinicians and patients should be aware that combined treatments are associated with considerable late side effects on bowel and anorectal functions, especially in terms of bowel frequency, urgency and fecal incontinence. Bruheim *et al*[125] explored long-term morbidity and QoL after radiotherapy (50 Gy) and total mesorectal excision (TME) for rectal cancer in a national cohort of 535 Norwegian patients. The study showed that radiaton-treated patients experience considerably worse long-term effects on anorectal function (in terms of bowel frequency and incontinence) compared with non-radiation-treated patients with an impaired QoL.

Laparotomy vs laparoscopy for TME

Laparoscopic rectal surgery is an advanced major procedure and should be performed in dedicated centers by highly trained surgeons in both elderly and younger patients[126]. No randomized trials have explored the dif-

ferences in short- or long-term outcomes, functional results or QoL specifically in the elderly population, as the mean age of the patients included in those studies has been shown to be not over 69 years of age[127]. Of the few dedicated studies, Akiyoshi *et al*[128] cleared the way for additional and more structured, multicenter trials. They prospectively analyzed a single center in which 315 patients were operated on for rectal cancer from 2001 to 2008. A comparison was carried out regarding 44 patients over 75 years of age who underwent laparoscopic TME (Group A), 228 over 75 years of age who underwent the same procedure (Group B) and 43 patients over 75 years of age who had their TME performed in the standard open fashion (Group C). Both the oncological results (distal margins, circumferential margins and number of lymph nodes retrieved), and the postoperative morbidity and mortality did not statistically differ in the three groups despite significant differences in the ASA score. The restoration of bowel function and length of stay were both in favor of the laparoscopic group ($P < 0.0001$ and $P < 0.002$, respectively), reinforcing the benefit of a laparoscopic approach. Furthermore, elderly patients actually seemed to benefit more from the laparoscopic approach in terms of postoperative cardiovascular and pulmonary complications[128]. No conclusive assumption could be drawn regarding this topic but the available evidence seems to show that laparoscopy, when performed in high volume centers, is feasible and effective for elderly patients with rectal cancer[124].

Habr-Gama effect

Neoadjuvant chemoradiation treatment (CRT) has been shown to be responsible for significant tumor regression and local recurrence rate reduction[129,130]. The result of medical treatment has been so remarkable that Dr. Habr-Gama set the bar at a higher level and decided not to operate on patients having a complete clinical response (cCR) after CRT[131]. The same group of scientists has recently published a paper on watchful waiting in a series of 70 patients with cT2-4, cN1-2 low rectal cancer who underwent extensive CRT (54 Gy + 6 cycles of 5-fluorouracil and leucovorin)[132]. Of the 47 patients with a complete clinical and radiological response, 8 (17%) experienced an early recurrence after 16-50 wk of follow-up. Late recurrence was instead recorded in 4 out of 39 patients with a cCR after 13-35 mo from CRT. All these patients underwent R0 radical surgery; no recurrence was recorded after 25.5 mo of mean follow-up. Overall, 35 patients (51%) did not require any surgical treatment and they were free from disease after 56 mo of a median follow-up. The mean age of the patients in the study was 60.2 ± 12.9 years old; thus, the study was not specifically addressed to elderly patients.

Despite the lack of focus on rectal cancer, in senior adults, this might be an intriguing solution for patients considered unfit for surgery after a multidimensional/multidisciplinary assessment. The difference from the past is that this will not be considered a palliative solution but standard treatment with perhaps more than a 50% chance of curing frail elderly patients with rectal cancer. Following the same pathway, the same group designed a different approach for those patients who partially responded to CRT (ypT0-2, N0) and they performed transanal endoscopic microsurgery (TEM) in 27 patients to partially remove the rectal wall (containing the cancer) instead of classic TME radical surgery[133]. Nine patients had a recurrence after a median follow-up of 15 mo (5 with exclusively systemic relapse and 4 with local relapse). The TEM specimens of 3 patients had shown ypT2 cancer while one patient with local recurrence was previously staged as ypT1. At univariate analysis, initial tumor size and lymphovascular invasion were found to be associated with local recurrence while, in the multivariate analysis, only the lymphovascular invasion remained (OR = 21.9; 95%CI: 1.3-362.9) statistically significant. The conclusion by the authors, subsequently emphasized by other reviewers, was a "word of caution" on both patient selection[134] (choosing the patients with no cCR is equivalent to choosing those with the highest risk of not surviving) and the treatment itself. Again, the study was clearly not designed for elderly patients (perhaps unfit for major surgery) but it should be considered an interesting start within a promising application regarding frail elderly patient care.

POSTOPERATIVE RECOVERY AFTER CRC SURGERY IN THE ELDERLY

After surgery, the functional recovery of elderly patients is defined as the ability to regain physical mobility, feeding capacity (swallowing, bowel function, performing the necessary movements to bring the food to the patient's mouth) and the attitude of being independent in the activities of daily living. Postoperative memory loss and delirium after general anesthesia and hospitalization have also been widely feared by elderly patients and their caregivers. Several attempts have been made to reduce the risk of postoperative delirium but, unfortunately, no effective strategies have been identified. In a recent study, Hempenius *et al*[135] designed a dedicated geriatric multidisciplinary approach for patients with solid cancer. Unfortunately, the randomized trial failed to demonstrate any advantage in patients who were treated with a multimodality approach compared with standard care. Several strategies have been promoted in order to achieve early functional capacity after major oncological surgery, beginning with the preoperative period, continuing with less invasive surgical techniques and, subsequently, postoperative strategies.

Laparoscopic approach and independence

The laparoscopic approach for CRC elderly patients has previously been discussed. Two additional papers are mentioned as examples. The first is by Frasson *et al*[107] who specifically focused on functional recovery after laparoscopic surgery and the specific benefits for the elderly. They analyzed a series of 535 patients with colorectal dis-

ease randomly assigned to laparoscopic (n = 268) or open (n = 267) resection. The CRC patients represented 78.5% of the entire sample (n = 420). Within the two groups, the outcomes of young patients (under 70 years of age) were compared with those obtained in patients over 70 years of age. The authors concluded that laparoscopy should be considered as the first option in elderly patients as it improves the preservation of functional status permitting a higher rate of postoperative independence at discharge and faster postoperative recovery. Notable advantages obtained from a laparoscopic approach compared with open surgery were ultimately more pronounced among the elderly than in younger patients. Stocchi *et al*[106] were also able to demonstrate that independent status at admission (assessed in 37 patients undergoing laparoscopic-assisted colectomy and 38 undergoing open colectomy) was more frequently maintained at discharge in those undergoing laparoscopic-assisted colectomy (95% *vs* 76%, respectively, P = 0.025).

Rapid rehabilitation program

As is well known, fast-track programs include preoperative patient education, no routine bowel preparation, minimal perioperative starvation, early removal of the nasogastric tube and urinary catheter, tailored anesthesia and postoperative analgesia, early postoperative diet intake and mobilization with minimal fluid infusion. The literature suggests that elderly patients have an advantage in functional recovery if enrolled in a fast-track program. Baek *et al*[136] analyzed a group of 337 patients (87 over 70 years of age and 250 under 70 years of age) who underwent laparoscopic colorectal surgery with a perioperative fast-track program. No significant differences were observed in terms of return of flatus, stool passage, progression of diet, complication rate (26% in the elderly patients *vs* 32% in the young patients) and length of hospital stay (12 d for each group). These results were obtained regardless of a significant differences between the two groups when considering age, presence of comorbidities (70% in the elderly *vs* 44.7% in the younger patients) and ASA score. In particular, they observed a lower than expected cardiopulmonary complication rate which they acknowledged was most likely due to the use of a low-pressure pneumoperitoneum (8 mmHg). The only significant differences were observed in readmission rate and emergency room visits (11.7% *vs* 4%, respectively).

Pawa *et al*[137] achieved similar results, with a median length of stay of 6 d for a 558 patient group under 80 years of age while a total of 8 d was recorded in a cohort of 130 patients 80 years of age or older (P = 0.363). No significant differences in 30-d readmission rate (8.6% of the whole population) were observed in the study. Senagore *et al*[101] compared the benefits of an open *vs* a laparoscopic colectomy among elderly (≥ 70 years old) and young patients (< 60 years old) in a fast track program [Controlled Rehabilitation with Early Ambulation and Diet (CREAD) program], and concluded that the association of CREAD and the laparoscopic technique gives better results in terms of length of stay, hospital costs, readmission rate and reoperation rate for both elderly and young people. Similar results were reported by Keller *et al*[138] who prospectively analyzed a group of 302 patients under 70 years of age compared with a group of 153 patients over 70 years of age. Wang *et al*[139] randomly divided an elderly patient group undergoing laparoscopic colon resection into a fast-track rehabilitation group (n = 40) and a conventional care group (n = 38); they concluded that the main advantages were a shorter length of hospital stay and a lower complication rate for patients in the fast-track group. We can conclude that fast track protocols are not only feasible but they also have notable advantages in elderly patients compared with younger patients. Elderly cancer patients greatly benefit from the avoidance of bowel preparation (associated with hydroelectrolyte imbalances) and opioid restriction (associated with ileus, nausea and vomiting). Furthermore, encouraging early ambulation avoids the risk of prolonged bed rest.

Considerations regarding QoL

Personalized treatment for elderly patients with CRC include not only the main goal of obtaining prolonged survival but also the achievement of a satisfactory QoL. Few studies have analyzed the QoL after surgery for CRC in senior adults. Mastracci *et al*[140] administered a generic test (Short Form-36) and two specific questionnaires to measure the QoL after medical and surgical treatment for CRC (EORTC QLQ-CR38 and EORTC QLQ-C30) to 29 Canadian patients (mean age, 83.2 ± 2.79 years). The goal was to obtain data regarding their physical function, body pain, social functioning, vitality and general health perception. Only patients who were able to complete the questionnaire were included in the study (possible bias) and a comparison was made with a similar group of randomly chosen 65-70-year-old patients (n = 29). There was no statistically significant difference between the groups in mean scores for body image, future perspective, sexual function/enjoyment, gastrointestinal symptoms and weight loss. The domains which differed significantly among the two groups were physical functioning, functional role, micturition, and stoma-related problems. Authors ascribed these differences to natural senescence, with the exception of stoma-related problems.

An interesting prospective multicenter study by Scarpa *et al*[141] analyzed the QoL of elderly *vs* younger patients undergoing colorectal surgery. A total of 116 patients were enrolled in this study: 33 patients over 70 years of age had a laparoscopic colectomy whereas 24 had an open colectomy; 44 patients under 70 years of age had a laparoscopic colectomy and 15 of them had an open colectomy. They used three questionnaires regarding generic (EORTC QLQ-C30) and disease-specific QoL (EORTC QLQ-CR29), and treatment satisfaction (EORTC IN-PATSAT32). They showed that elderly patients undergoing a laparoscopic colectomy for can-

cer experienced fewer postoperative local complications than elderly patients undergoing an open colectomy. Nevertheless, in the first postoperative mo, these patients experienced a poorer QoL compared with younger patients undergoing the same surgery ($P = 0.003$), with impairment of all functions and the presence of fatigue, sleep disturbance, appetite loss and shortness of breath. In the laparoscopic elderly patient group, there were no significant differences in satisfaction or QoL, despite a lower postoperative complication rate compared with the elderly open surgery group. Finally, Amemiya *et al*[142] prospectively analyzed 223 patients over 75 years of age operated on for CRC ($n = 132$) and gastric cancer ($n = 99$). They administered the Short Form-12 and EuroQoL 5-D tests at 1 wk, 1 mo, 3 mo and 6 mo after surgery. The QoL measured at 1 wk and 6 mo showed a significant improvement ($P < 0.005$). Functional recovery and activities of daily living status improved after surgery in the majority of patients; however, a temporary or prolonged decline in recovery was found in those who developed postoperative complications.

CONCLUSION

Aging of world populations is occurring, and especially in Western countries. Becoming old means being less and less independent from a number of perspectives. Among the various causes leading to a decrease in functional capacity, declining health plays a pivotal role. Aging in the populations of Western countries is becoming one of the most significant challenges for our health care systems. Elderly patients have multiple comorbidities, and unpredictable social and family situations; when cancer is diagnosed, this adds to in an already complicated situation. Among the elderly, those who are vulnerable or even frail are the ones who really deviate from the standard curves.

Despite aging in Western countries and the clear challenge for healthcare professionals and scientists, few studies have specifically been designed to assess the success of care strategies in this cohort of patients. Elderly people do not fit into randomized control trials and, in many cases, the results obtained from observation studies (as often happens in elderly population) are considered level B/C evidence by the scientific community. This is quite surprising if we consider one of the most frequent causes of cancer-related death in the elderly population: colorectal cancer. Why should we focus our attention on complicated, demanding, unconventional, non-reducible-to-the-standard-practice type of patients who are historically considered less amenable to curative treatment because of their age? The elderly in Western countries who have CRC have a worse prognosis than younger patients; but this is true only during the first 12 mo after surgery while 5-year cancer-related survival does not differ from the rest of the population which is healthier and has access to more sophisticated treatment. We have to focus our attention on that period of time. Our review showed that, as physicians, the only answer we can give is to implement strategies for personalizing the treatment of the elderly with cancer. Individualized care does not mean being subjective. Many studies have defined rigorous pathways, screening tools and tailored surgical and postoperative strategies in order to obtain this goal. The multidimensional/multidisciplinary approach is the key for rejecting "the gut-feeling type of decision" and for promoting optimal individual patient care. Our review showed how sarcopenia (measured both directly and indirectly with TUG or a 6-min walk test) seems to be the best predictor for postoperative outcomes. Prehabilitation, despite the lack of large randomized clinical trials, has been shown to be a promising start in reducing the most worrisome complication for an elderly individual: the loss of independence. At the same time, less invasive surgery is being implemented in order to reduce pulmonary and cardiologic complications and eventually the length of stay, such as the advanced laparoscopic approach. During the postoperative period, fast track strategies are extremely beneficial for the elderly who have shown positive results with reduced amounts of opioids, early mobilization and oral feeding. Intriguing solutions have also been described for a non- or local-surgical approach to low rectal cancer and, despite the lack of specific trials, it could be an interesting solution to be offered to frail individuals who cannot undergo a standard approach. Therefore, why should we treat these challenging, complicated, demanding, unconventional elderly patients with cancer? This review cannot provide the profound answer that we need to give as physicians and human beings. This study was carried out to reveal the evidence in the current literature in order to help whoever decides to assist these frail patients and devote their professionalism to rediscovering the true essence of Medicine: personalized care for the patient.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Inhibition of host immune response in colorectal cancer: Human leukocyte antigen-G and beyond

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Abstract

Colorectal cancer (CRC) is one of the most diffuse cancers worldwide and is still a clinical burden. Increasing evidences associate CRC clinical outcome to immune contexture represented by adaptive immune cells. Their type, density and location are summarized in the Immune Score that has been shown to improve prognostic prediction of CRC patients. The non-classical MHC class I human leukocyte antigen-G (HLA-G), is a crucial tumor-driven immune escape molecule involved in immune tolerance. HLA-G and soluble counterparts are able to exert inhibitory functions by direct interactions with inhibitory receptors present on both innate cells such as natural killer cells, and adaptive immune cells as cytotoxic T and B lymphocytes. HLA-G may play a prominent role in CRC strategies to avoid host immunosurveillance. This review highlights the current knowledge on HLA-G contribution in CRC, in related inflammatory diseases and in other type of cancers and disorders. HLA-G genetic setting (specific haplotypes, genotypes and alleles frequencies) and association with circulating/soluble profiles was highlighted. HLA G prognostic and predictive value in CRC was investigated in order to define a novel prognostic immune biomarker in CRC.

Key words: Colorectal cancer; Human leukocyte antigen-G; Immune score; T lymphocytes; Untranslated regions

Core tip: Colorectal cancer (CRC) prognosis is strictly associated with the immune contexture of tumor microenvironment. IS improves prognostic prediction in CRC. Human leukocyte antigen-G (HLA-G) through its direct inhibitory functions on NK cells and cytotoxic T and B lymphocytes represents a crucial tumor-driven immune escape molecule. This review highlights the current knowledge on HLA-G in CRC and in related inflammatory diseases. HLA-G genetic setting and circulating/soluble profiles need to be defined to comprehend CRC strategies to avoid host immune defences. We suggest that HLA G could represent a novel prognostic immune biomarker to associate with the Immune Score to better characterize host immune response in CRC.

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INTRODUCTION

Colorectal cancer (CRC) remains one of the leading causes of cancer death worldwide[1-3]. CRC develops

sporadically[4], in the setting of hereditary forms[5], or on the basis of inflammatory bowel disease (IBD)[6]. The adenoma-carcinoma transition is the well-established known model for CRC onset[7] and, its genetic and molecular background have been widely described[5,8]. In the last years, the elaborate exchange among innate-adaptive immune cells of the tumor microenvironment, the local inflammatory state, and the host immune response in solid tumors, generated the concept of cancer immunoediting, characterized by the final escape phase exerted by the cancer from host defence immunity[9]. Increasing evidences demonstrated that solid tumors such as CRC are infiltrated by different adaptive cells of the immune contexture that may influence the progression of the disease[10]. These experimental observations finally provided the design of the Immune Score (IS) that is now considered as a novel and independent prognostic marker, in human cancer as well in CRC[11]. IS, is represented by densities of adaptive immune cells: infiltrating $CD8^+$ cytotoxic T lymphocytes (CTLs), and $CD45RO^+$ memory T cells, detected in center and marginal tumor areas[12]. Higher number of infiltrating $CD8^+$ CTLs and $CD45RO^+$ memory T cells correlates with an improved patient prognosis; lower numbers correlate with tumor relapse[13]. IS demonstrated to have a prognostic value for overall survival (OS) and disease free survival (DFS) in retrospective studies[14], and is currently being submitted for clinical validation in prospective CRC studies, in 16 different Countries world wide[15].

The non-classical HLA-G is considered a tolerogenic molecule due to its inhibitory functions *vs* T lymphocytes, NK cells and other cell types of immune contexture[16]. HLA-G is only recently been involved in tumor escape mechanisms from the host immune recognition and destruction[17], and is considered a tumor microenvironment molecule[16,18]. HLA-G shows a lower nucleotide variability in coding sequences, while is highly polymorphic in the untranslated regions (UTRs), both in 5' and 3' segments[19,20] (Figure 1). Polymorphic sites mainly present in the 3'UTR region, may affect the post transcriptional regulation and biological functions of HLA-G[21]. Indeed, through alternative splicing, HLA-G can be expressed as seven different and specific molecules, four membrane bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7)[22]. Most of the published data concern the HLA-G1 molecule and its soluble counterpart HLA-G5[23]. HLA-G1 can be shed generating a soluble isoform (sHLA-G1)[17]. The functional characterizations of the five remaining HLA-G isoforms have been yet not clearly elucidated. HLA-G is over-expressed in CRC[24-26] and is a common signature in other types of cancer, autoimmune disorders, viral infections and transplantations[16-18]. Increase in circulating sHLA-G has been detected in CRC in few studies[27,28] and in other malignancies[23,29-31]. Growing attention is focusing on the genetic setting related to the UTRs, especially the 3'UTR involved in micro RNA (miRNA) binding[32]. Single nucleotide polymorphisms (SNPs) in this region have been associated with the disease risk in cancer[33-35] and other disorders[36,37] (Table 1), but the association with CRC and the prognostic value in this type of malignancy, remain to define. Aim of this review was to investigate the HLA-G as a potential prognostic biomarker in CRC and related inflammatory colon diseases, both at the genetic and circulating profiles (Table 2). Genotypic-phenotypic correlation has been highlighted and its potential role in IS explored (Figure 2).

CRC AND CHRONIC COLONIC INFLAMMATION

CRC is one of the most diffuse cancers worldwide with about 1.2 million new cases and 600000 deaths recorded annually[1].

Despite improvements and advances in diagnosis, surgery and treatment, CRC is still the 2nd most common cause of cancer death in the United States and other industrialized countries[2,3].

Development of sporadic (88%-94%) and hereditary forms of CRC are mainly related to the accumulation of genetic changes in gatekeeper and caretaker genes like *APC* and other oncoproteins (*K-ras*, *erb-s*, *c-src*, *β-catenin, PI3K*) and tumor suppressors (*p53*, *Smad4*)[4,5], according to the aberrant crypt foci (ACF)-adenoma-carcinoma transition model[7]. It is well-established that the acquisition of these mutations in a multistep mechanism is part of the genomic instability process that comprehends the chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways[8]. While CIMP exhibits gene silencing due to hypermethylation of CpG islands, in CIN positive tumors (about 70% of sporadic CRCs) an imbalance in chromosome number (aneuploidy), subchromosomal genomic amplifications and a high frequency of loss of heterozygosity (LOH) are observed[38]. About 15% of sporadic CRCs, mostly in the proximal colon anatomic site, are characterized by MSI with a large number of mutations at microsatellite sequences interesting the DNA mismatch repair (MMR) system, so the consequence is the accumulation of thousands of unrepaired mutations[39,40].

Genesis of most of CRCs depends also from environmental factors like intestinal microbiota, dietary habits and lifestyle, associated with the patient genetic background[41]. The chronic colonic inflammation due to active ulcerative colitis (UC) or Crohn's disease (CD), the two major forms of inflammatory bowel disease (IBD), lead to the colitis-associated cancer (CAC) development that is a CRC subtype[6]. In UC, inflammation is limited to the mucosal layer and usually starts from the rectum spreading then into the colon, while in CD all the layers of gut wall are interested with the terminal ileum and also the colon as the most common sites[42].

IBD subjects are at increased risk to develop the tumor; it is estimated that about 20% of patients affected by chronic UD and CD for a long time, within 30 years, develop CAC; thus CRC risk increases with the duration

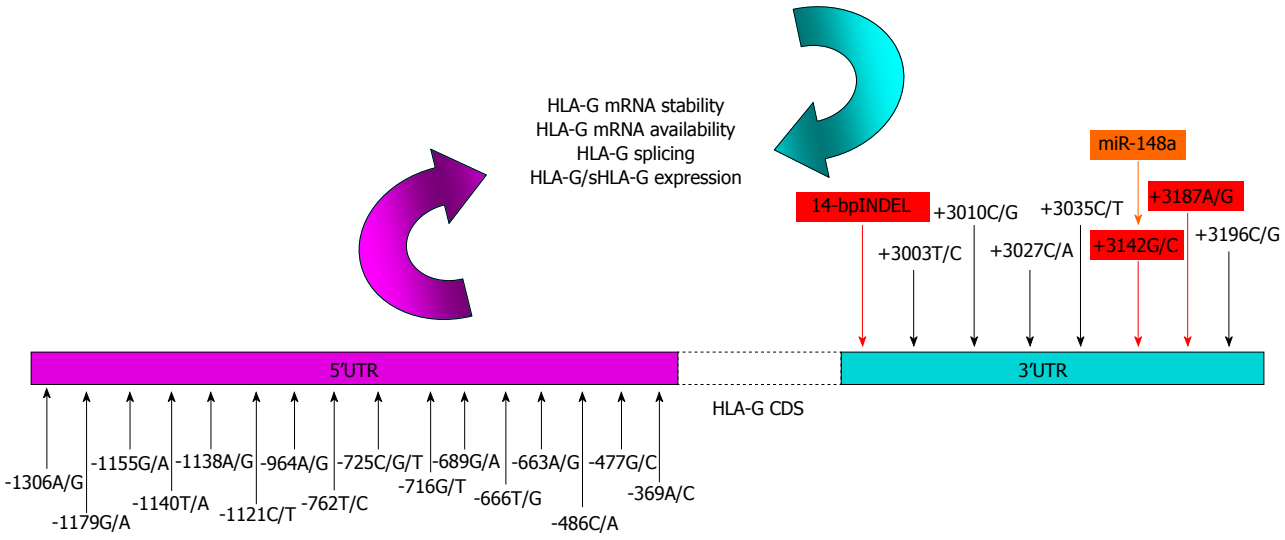

Figure 1 Human leukocyte antigen-G single nucleotide polymorphisms involved in the biological features of the protein: nucleotide variants in the 5'-3' untranslated regions may influence human leukocyte antigen-G expression levels by modifying the affinity of gene targeted sequences for transcriptional (5') or post-transcriptional (3') factors respectively. Polymorphisms in 5'UTR (fucsia) were previously described by Costa *et al*[113] those in 3'UTR (light blue) by Castelli *et al*[19]. In red 3'UTR SNPs involved in HLA-G mRNA stability and availability are highlighted. In orange the only one microRNA with a demostrated functional inhibitory role in the *HLA-G* expression[134] was highlighted . UTR: Untranslated region; SNPs: Single nucleotide polymorphisms; HLA-G: Human leukocyte antigen-G.

Table 1 List of associations found between single nucleotide polymorphisms/alleles in human leukocyte antigen-G untranslated regions and different pathologies; genotype-phenotype correlations were also reported

UTRs	SNP	Genotype and/or allele	Disease	Association with[1]	UTR SNP and sHLA-G correlation	sHLA-G level	Statistical significance[2]	Country	Ref.
3′	14 bp INDEL	14 bp I/I	PE	Increased disease risk	ND	ND	Yes	China	[36]
3′	14 bp INDEL	14 bp I/I	RSA[3]	Increased disease risk	ND	ND	No	Denmark	[112]
3′	+3142 C/G	+3142 GG and G allele	SLE	Increased disease risk	ND	ND	Yes	Brazil	[37]
3′	14 bp INDEL	14 bp I/I	SLE	Increased disease risk	ND	ND	No	Brazil	[37]
3′	14 bp INDEL	14 bp I/I	OvaC	Increased disease risk	ND	ND	Yes	Canada	[33]
3′	14 bp INDEL	14 bp D/D	EsophC	Increased disease risk	ND	ND	Yes	China	[34]
3′	14 bp INDEL	14 bp D/D and D allele	HCC	Increased disease risk	ND	ND	Yes	Brazil	[35]
3′	14 bp INDEL	14 bp I/I and D allele	HCC	Increased disease risk	ND	ND	No	South Korea	[137]
3′	14 bp INDEL	14 bp I/I	Allo-HSCT	Lower OS and DFS	ND	ND	Yes	Italy	[138]
3′	14 bp INDEL	14 bp D/D	RA	MTX therapy (responder group)	Yes	Higher	Yes[4]	Italy	[114]
3′	14 bp INDEL	14 bp I/I	RR-MS	sHLA-G	Yes	Lower	Yes[4]	Italy	[139]
3′	+3142 C/G	+3142 GG	RR-MS	sHLA-G	Yes	Lower	Yes[4]	Italy	[139]
3′	14 bp INDEL	14 bp I/I	IVF[3]	sHLA-G	Yes	Absent	Yes	Denmark	[141]
5′	-725C/G/T	-725C>G	IVF[3]	sHLA-G	Yes	Absent	ND	Denmark	[141]
3′	14 bp INDEL	14 bp I/I	Heart T	sHLA-G	Yes	Lower	Yes	Canada	[142]
3′	14 bp INDEL	14 bp I/I	HD	sHLA-G	Yes	Lower	Yes	China	[143]
3′	14 bp INDEL	14 bp D allele	ERA	Improved disease remission	Yes	Higher	Yes[4]	Italy	[144]
5′	-725C/G/T	-725Callele	RPL[3]	sHLA-G	Yes	Lower	Yes	Iraq	[148]
3′	14 bp INDEL	14 bp I/I	PTC	Increased disease risk not found	No	Higher	Yes[5]	Italy	[31]

[1]Data reported in this column are related to the association with SNPs in UTRs; the increased disease risk was compared with a HD control group; [2]Statistical significance is referred to HLA-G SNP and disease status analysis; [3]The disease status is related to infertility problems; [4]A statistical significance was found also between HLA-G SNP and sHLA-G levels; [5]A statistical significance was found between sHLA-G levels and the disease risk. UTR: Untranslated region; SNP: Single nucleotide polymorphism; sHLA-G: Soluble human leukocyte antigen-G; INDEL: Insertion/deletion polymorphism; I: Insertion; D: Deletion; PE: Pre-eclampsia; RSA: Recurrent spontaneous abortions; SLE: Systemic lupus erythematous; OvaC: Ovarian cancer; EsophC: Esophageal cancer; HCC: Hepatocellular carcinoma; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; RA: Rheumatoid arthritis; RR-MS: Relapsing-remitting multiple sclerosis; IVF: *In vitro* fertilization failure; Heart T: Heart transplantation; HD: Healthy blood donors; ERA: Early rheumatoid arthritis; RPL: Recurrent pregnancy loss; PTC: Papillary thyroid carcinoma; OS: Overall survival; DFS: Disease free survival; MTX: Methotrexate; ND: Not determined.

of the IBD and the severity of inflammation[43].

Pathogenesis of CAC in chronic colitis patients differs from the classical model sustained by sporadic CRC, for a transition from low-high grade dysplasia to carcinoma and also for the sequence of cellular and molecular events[44]. In CRC adenomatous polyposis coli (APC)

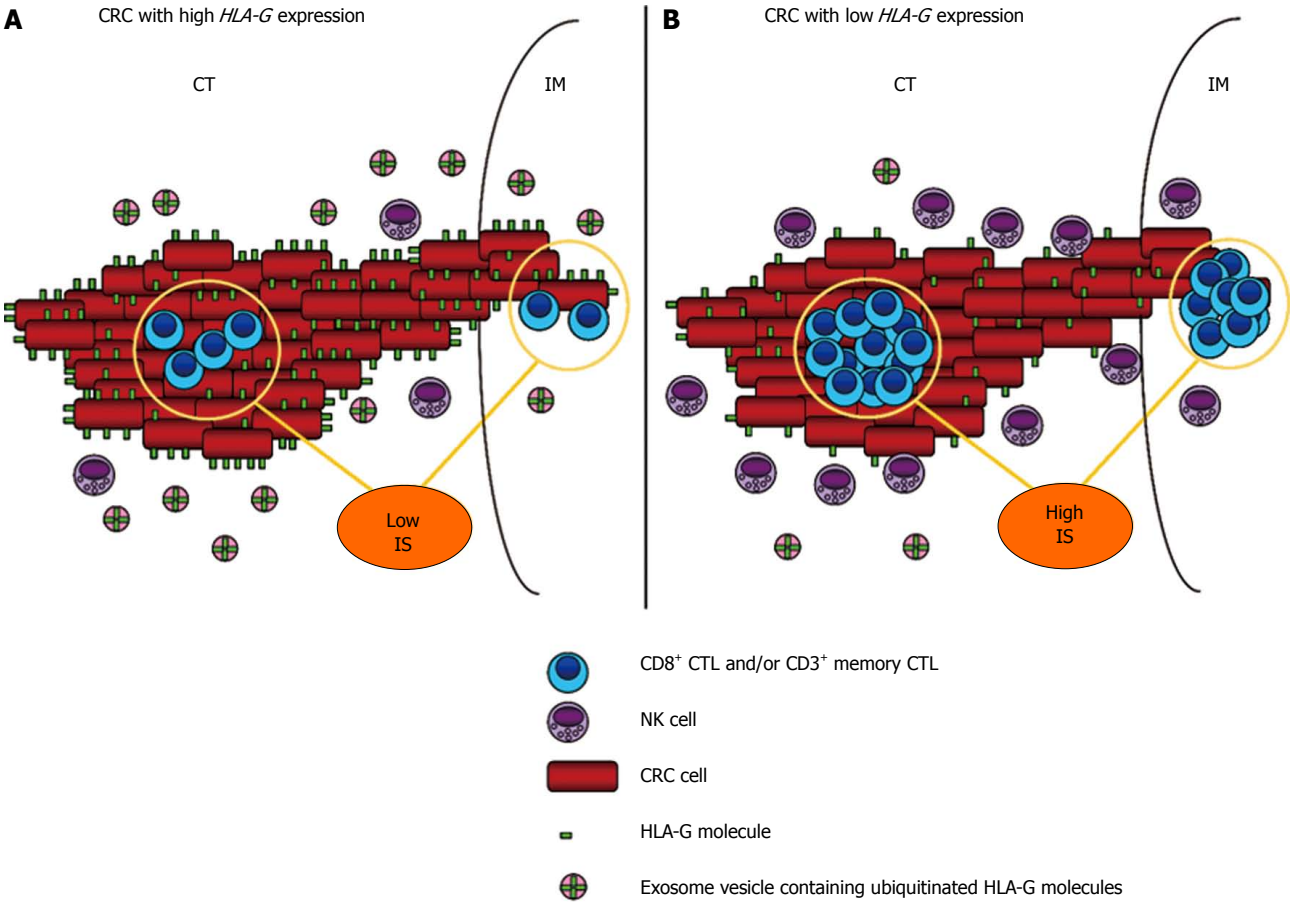

Figure 2 Schematic representation of colorectal cancer tumor microenvironment: potential influence of human leukocyte antigen-G expression on Immune Score and host immune local response. A: High *HLA-G* expression shows an inhibitory role against host immune defences, represented mostly by natural killer cells and CTLs. Increased *HLA-G* expression in the local microenvironment is also supported by exosome contribution through a hypothetical trogocytosis release mechanism that mediates HLA-G up-take. Another consequence is a direct association with a low IS value due to HLA-G inhibitory functions on CTLs in CT/IM areas of the tumor, and consequently with a worse patient prognosis (not represented); B: Lower HLA-G expression correlates with a high IS value and a favoureable immune contexture that improves host immune response and patient prognosis (not represented). Representation of IS is referred to recent quality and validation criteria[15] (see text for further clarifications). HLA-G: Human leukocyte antigen-G; CTLs: Cytotoxic T lymphocytes; IS: Immune Score; CT: Center of the tumor; IM: Invasive margin.

protein loss of function, is an early event during the formation of precocious adenoma, while it is less frequent and occurs in the late pathogenesis of CAC[45]. p53-loss of heterozygosity (LOH), *p53* mutations or loss of function are early molecular events characterizing CAC origin instead of CRC in which they frequently occur in the late adenoma-carcinoma transition[46,47].

IMMUNE CONTEXTURE IN INFLAMMATION AND CRC

The chronic inflammatory status is a common feature of colorectal and colitis-associated tumors. Chronic inflammation is modulated by immune innate/adaptive cells infiltration and immune microenvironment[48,49]. The crucial role of inflammation in tumorigenesis is emerged only recently and now it is estimated that about 15%-20% of cancers are related to an underlying chronic inflammation process[50,51]. CAC is considered a classical inflammation-driven cancer as demonstrated in mice models in presence of dextran sodium sulphate (DSS) and the procarcinogen azoxymethane (AOM)[52].

A growing body of evidence is focusing both on relationships between the immune contexture of solid tumors like CRC, and patient prognosis in terms of DFS and OS[53]. Tumor microenvironment is represented by a complex network of stromal, inflammatory and immunocompetent cells. Histopathological analyses show that solid cancers are infiltrated by innate-adaptive immune cells, that have a role in tumor growth[54,55].

The interplay among tumor cells and immune system is highly complex and suggested the concept of cancer immunoediting, a dual process in which the main actors are the host-protective and the tumor-promoting actions of immunity[9,56,57]. Cancer immunoediting occurs in three phases: elimination, equilibrium and escape. In the elimination phase (the modern concept of the older notion "cancer immunosurveillance")[58], innate and adaptive immunity work together to recognise and destroy nascent tumor cells. If tumor cell variants are not completely eliminated, will enter into an equilibrium phase, in which adaptive immunity controls and stems the growth of

Table 2 Summary of human leukocyte antigen-G evaluations in colorectal cancer and related colonic diseases of the gastrointestinal tract

Sample type	HLA-Gs	Methods	Disease, *n*	Relevances	Ref.
Tumor DNA	HLA-G	RT-PCR	CRC, *n* = 39	HLA-G mRNA was significantly more expressed in CRC (87.2%) than in the extra neoplastic tissue	[24]
Tumor tissue	HLA-G	IHC	CRC, *n* = 201	HLA-G is over-expressed in primary CRC sites (64.6%), but not in the normal CRC tissues or benign adenomas	[25]
Tumor tissue	HLA-G	IHC	UC, *n* = 24; CD, *n* = 19	HLA-G and IL-10 are highly expressed in UC but not in CD tissue biopsies	[154]
Tumor tissue	HLA-G	IHC	CRC, *n* = 60; DA, *n* = 67; BC, *n* = 37; AC, *n* = 52	HLA-G is over-expressed in 52 % of CRC lesions and also in 79% of PDAs, 76% in BC and 75% AC	[26]
Tumor tissue	HLA-G	IHC	CRC, *n* = 415	HLA-G is expressed in > 30% of CRC lesions (data summarize published data collected until 2008)	[16]
Tumor tissue	HLA-G	IHC	CRC, *n* = 154	HLA-G is expressed in > 30% of CRC lesions (data summarize published data collected until 2005)	[17]
Serum	sHLA-G	ELISA	CRC, *n* = 144	Higher sHLA-G levels in CRC (median 124.3 U/mL) compared to benign colorectal diseases (cut off value 88.6 U/mL). CEA showed less sensitivity e specificity	[27]
Plasma	sHLA-G	ELISA	CRC, *n* = 37	sHLA-G as a diagnostic biomarker for the detection of early CRC (median 84 U/mL) with respect to BD (median 34 U/mL)	[28]
PBMC	sHLA-G	ELISA	HD, *n* = 30; CD, *n* = 10; UC, *n* = 18	Spontaneous secretion of sHLA-G from cultured PBMCs of CD but not in UC and BD Secretion of sHLA-G in CD patient cultures and BD but no in UC, after LPS stimulation	[161]
Plasma and PBMC	HLA-G	ELISA	UC, *n* = 27; CD, *n* = 22	Immunosuppressive therapy decreases sHLA-G hyperproduction in CD and induces its release in UD, in both plasma and in PBMC culture supernatants	[162]

HLA-G: Human leukocyte antigen-G; sHLA-G: Soluble HLA-G; CRC: Colorectal cancer; UC: Ulcerative colitis; CD: Crohn's disease; PDA: Pancreatic ductal adenocarcinoma; BC: Biliary cancer; AC: Ampullary cancer; HD: Healthy blood donors; PBMC: Peripheral blood mononuclear cells; IHC: Immunohistochemistry; LPS: Lipopolysaccharide; CEA: Carcinoembryonic antigen; IL: Interleukin; ELISA: Enzyme-linked immunosorbent assay.

clinically undetectable tumor cells and blocks tumor cell immunogenicity. When dormancy of tumoral cells stops, malignant cells with reduced immunogenicity shift into the escape phase and begin to grow and proliferate in an immunologically unrestrained way, establishing an immunosuppressive tumor microenvironment, and becoming clinically detectable. Escape mechanism from immune host control is now considered one of the hallmarks of cancer[59]. Innate immunity is the first line of host defense and it is specialized in counteracting cancer cells and virally infected cells.

Innate immune cells *i.e.*, myeloid derived suppressor cells (MDSCs), macrophages, neutrophils, dendritic cells (DCs), mast cells (MCs), and NK cells, may have a pro- or anti-tumorigenic role in both CRC and CAC[60]. MDSCs under the control of nuclear factor κB (NF-κB), produce interleukin (IL)-6 that activates transcription factor signal transducer and activator of transcription 3 (STAT3) resulting in survival, growth and progression signals in early CAC[61].

Macrophages are responsible mainly for pro-inflammatory cytokines release and are distinguished in two types. Type 1 macrophages (M1) that are activated by interferon (IFN)-γ or tumor necrosis factor (TNF) cytokines, are efficient producers of reactive oxygen and nitrogen species, have an interleukin (IL)-10^{low} IL-12^{high} IL-23^{high} inflammatory phenotype, and tend to negatively control tumor growth[62]. Conversely, type 2 macrophages (M2) share an IL-10^{high} IL-12^{low} IL-23^{low} anti-inflammatory phenotype and stimulate cancer proliferation by secreting immunosuppressive cytokines like IL-10. Production of mediators promoting angiogenesis such as Vascular Endothelial Growth Factor A (VEGFA) and Cyclo-Oxygenase-2 (COX-2)-derived prostaglandin E2[63], hypoxia-dependent upregulation of chemokines (C-X-C motif), induces accumulation of M2 macrophages that is the predominant phenotype in tumor microenvironment[64,65]. A macrophage polarization of tumor-associated macrophages (TAMs) due to the local colonic microenvironment during tumor progression is observed with a switching from M1 (inflammatory) to M2 (anti-inflammatory) type and a gradual NF-κB inhibition[66]. TAMs are one of the major components of leukocyte infiltrates in tumors and represent an independent prognostic factor of poor prognosis in multivariate analysis in different malignancies[67]. In CRC, TAMs correlate with improved OS[68]. Finally, innate immune cells orchestrate a complex inflammatory environment that may be modulated to either stimulate or inhibit CRC proliferation[69,70].

While innate immunity does not involve a specific antigen or peptide or particular tumor-associated antigen recognition, this is a prerequisite for adaptive immunity. Cells of adaptive immune system are mainly represented by B lymphocytes, T helper 1 (Th1) and T helper 2 (Th2) CD4$^+$ cells, CD8$^+$ Cytotoxic T lymphocytes (CTL) cells and CD4$^+$ T regulatory (Treg) cells[71]. Recent findings of memory responses by NK cells suggest that also NK may contribute to adaptive immunity[72]. To destroy cancer cells, CTLs need to recognize an antigen exposed on the tumor cells in association with the human leukocyte antigen (HLA) class I proteins[73]. Only through recognition of this tumor cell antigen/HLA I complex for which

their T cell receptor (TCR) is specific, CTLs clonally expand and differentiate in memory T cells[74] ($CD45RO^+$). $CD45RO^+$ comprise $CD3^+$, $CD4^+$ and $CD8^+$ T cells that have been exposed to antigen and respond faster and with an increased intensity after antigen stimulation compared with naïve T cells[54].

Upon activation, CTLs release proteases and lytic components as perforin and mediate disruption of tumor cell membrane and activation of apoptotic pathway. $CD4^+$ T cells respond only to antigens presented by the HLA class Ⅱ proteins expressed by DCs in secondary or tertiary organs[75]. Many evidences highlight that one of the cancer immunoediting topic is due to the fact that T-cell recognition of tumor antigens drives the immunological destruction of nascent and developing cancer cells. One of the most well-known way used by tumors to escape from specific T-cell recognition mechanisms is down-regulation or total suppression of MHC I molecules, and specific alteration leading to the inefficient presentation of immunodominant antigens[76].

Recently, Matsushita *et al*[77], demonstrated that the immunoselection by $CD8^+$ T cells of tumor variants lacking strong tumor-specific antigens represents one of the mechanism by which cancer cells escape tumor immunity.

Th1 cells secrete cytokines like IFN-γ and TNF-α, support tissue destruction and CTLs by producing IL-2 required for $CD8^+$ proliferation[78]. Th2 cells produce cytokines such as IL-10, IL-4 and IL-5, and limit CTLs proliferation. Tregs that also highly express CD25 ($CD4^+CD25^+$) secrete IL-10 and Tumor Growth Factor (TGF)-β which dampen the immune response[79]. It should be emphasized that while CTLs, Th1 and Th17 inhibit cancer growth, Th2 cells and Tregs stimulate cancer proliferation. Moreover, a shift in Th1 (tumor rejection)/Th2 (tumor promotion) immune response is observed in CRC[80]. Restoring of normal immunological functions and pro-inflammatory cytokines after tumor resection in CRC were demonstrated, highlighting that CRC itself has a direct immunosuppressive effect[81,82].

Overall, immune contexture should be considered as comprising the density of $CD8^+$ CTLs and $CD45RO^+$ memory T cells, their location at the center of the tumor (CT) and invasive margin (IM), combined with the quality of tertiary lymphoid structures (TLS) and additional functionality entities such as Th1-related factors, chemokines, adhesion molecules and cytotoxic factors[83]. Indeed, in CRC, not only Th1 immunity markers (STAT1, IRF1, IFN-γ-SG pathway), cytotoxic markers (Granzyme Perforin, Granulysin, TIA1, Caspase pathway), but also chemoattraction (specific chemokines such as CX3CL1, CXCL10, CXCL9) and adhesion (molecules as ICAM1, VCAM1, MADCAM1) signatures are relevant in influencing the density of infiltrating immune cells[84,85].

CRC AND IMMUNE SCORE

Accumulating evidences since the late 1990s showed an association among tumor-infiltrating lymphocytes (TILs) able to inhibit cancer growth, and improved prognosis in CRC[10,86] and other malignancies such as melanoma[87] and ovarian cancer[88]. A particular phenotype in CRC is represented by MSI type tumors that are associated with a high TILs levels, loss or downregulation of HLA class Ⅰ, better patient prognosis, a reduced metastatic potential, and a different response to chemotherapy[39,60].

In 2005-2006 the idea that the adaptive immune response plays a role in preventing tumor recurrence in CRC emerged, mostly from the works of Pagès *et al*[89] and Galon *et al*[13]. The authors first demonstrated that CRCs with high density of infiltrating and effector memory T cells with a protective immune role, were less prompt to metastasize and can be associated with an increased survival of patients[89]. Subsequently, using the same cohort of patients, relationships among type, density and location of immune cells within the tumor and the clinical outcome, were investigated by using both genomic approaches and immunohistochemistry (IHC)[10]. Through the selection and the evaluation of expression levels of genes involved in inflammation, Th1 adaptive immunity and immunosuppression, Galon *et al*[13] found a dominant cluster of co-modulating genes for Th1 adaptive immune response (*i.e.*, IFNG, CD8a, GLNY, GZMB, CD3z). Applying specific tissue microarrays and a dedicated image analysis work station, a quantification of total ($CD3^+$) T lymphocytes, $CD8^+$ CTL effectors, associated molecule (GZMB), and $CD45RO^+$ memory T cells, was performed both in CT and IM.

High immune cell densities ($CD3^+$, $CD8^+$, GZMB and $CD45RO^+$) in both CT and IM tumor regions were present in CRC patients without recurrence after adjuvant therapy, while lower densities of the same immune cell types correlated with disease recrudescence. Results highlighted an inverse correlation among expression of these genes and CRC relapse suggesting that Th1 adaptive immunity improve clinical outcome[13]. Camus *et al*[14] demonstrated the association between loss of coordinated functional immune reaction and the progression of CRC to a metastatic phenotype. These preliminary results demonstrated for the first time that the host immune response plays an important role in determining the outcome of CRC patients. Type, density, and location of immune cells in CRCs increase the prediction accuracy of DFS and OS, and started to represent a superior and independent prognostic parameter with respect to the UICC-TNM well accepted classification[90].

Finally, all these data and evidences, culminated in the concept of "Immune Score" that emerged for the first time in 2011 in the work of Pagès *et al*[12]. A multivariate Cox proportional hazard regression model was used to assess the hazard ratio of the immune score combination (CD45RO/CD8) in specific tumor regions (CT/IM), together with clinical and histopathological tumor markers. Pagès *et al*[12], analyzing a large cohort of CRC patients with early (Ⅰ-Ⅱ) stage, showed that the combined analysis of cytotoxic ($CD8^+$) and memory ($CD45RO^+$) T cells confirmed its prognostic discrimina-

tory power in the prediction of tumor recurrence and survival[12]. Subsequently, the concept of IS as a clinical prognostic marker improving the standard TNM classification at any stage of CRC, has been established evaluating infiltrating lymphocytes of 599 CRC specimens by Mlecnik *et al*[91]. Patients with high IS had increased DFS and OS, and patients with a low IS were likely to experience a disease relapse[92]. IS represents a standardized, simple and powerful immune stratification system proposed as a novel prognostic immune marker for routine testing potentially helpful for CRC management to better identify and stratify high-risk patients who would benefit most from adjuvant therapy[12].

Cancer outcomes can vary significantly among patients with the same stage and this could be related to differences in immune cells densities from patient to patient as in CRC[93]. This argues the limit of traditional AJCC/UICC TNM classification in providing limited prognostic information and predicting response to therapy[94]. A worldwide task force representing 22 Institutions from 16 different countries, is working now for IS validation in clinical practise, with the aim to introduce it as a new component of classical cancer classification, that will maybe designate in future as TNM-I (TNM-Immune)[15]. To improve quality and validation in standard laboratories, IS will be quantified by the combination of the two easiest membrane stains CD3 and CD8 to avoid any background noise due to CD45RO and GZMB stains[15]. Special emphasis will be focused in the prognostic significance of validated immunologic parameters in CRC that with the primary goal to validate the prognostic power of the IS in routine settings of stage Ⅰ/Ⅱ/Ⅲ CRC patients and for recurrence prediction for stage Ⅱ CRC patients[95]. Thus, the bases to revise and renegotiate the clinical outcome based on classical clinical parameters have been seeded[96].

Recently, a great emphasis has been done in an attempt to define the prognostic role of another subtype of tumor infiltrating cells. Treg cells also positive for the nuclear transcription factor protein forkhead box P3 (Foxp3), showed a strong and independent prognostic significance in CRC, superior to $CD45RO^+$ and $CD8^+$ cells[97]. $Foxp3^+$ Tregs cells are generally associated with immunosuppressive properties and poor prognosis in different solid tumors such as hepatocellular[98], prostate[99] and pancreatic carcinoma[100], but conversely were reported to be associated with improved prognosis in CRC[97]. Although Foxp3 is a well accepted marker used to identify tumor infiltrating $CD4^+$ $CD25^+$ Tregs, it is known that a small proportion of $Foxp3^+$ cells may also be $CD8^+$. $CD8^+$ $CD25^+$ $Foxp3^+$ T cells showed suppressive capacities in CRC[101], suggesting that $Foxp3^+$ role should be more explored. Foxp3 expression evaluated in tumor cells was associated with worse outcome of patients in different solid tumors[102-104] but not in CRC, highlighting a new independent prognostic factor. Recently, $Foxp3^+$ expression in tumor cells was compared to $Foxp3^+$ Treg infiltration in CRC, demonstrating for the first time an inverse correlation between the number of $Foxp3^+$ Treg and the level of $Foxp3^+$ in tumor cells, suggesting an anti-proliferative effect of TGF-β on Tregs[105]. Furthermore, patients with high $Foxp3^+$ expression profile in CRC tumor cells were correlated with a poorer prognosis that was not observed for $Foxp3^+$ Treg in the tumor[106].

HLA-G: A CRUCIAL TUMOR-DRIVEN IMMUNE ESCAPE MOLECULE

HLA-G is considered as a tolerogenic molecule exerting its inhibitory functions by direct interaction with different inhibitory receptors of the immunoglobulin family present on NK cells (ILT2/CD85j, KIR2DL4/CD158d), T lymphocytes (ILT2/CD85j, KIR2DL4/CD158d), B cells (ILT2/CD85j), endothelial (CD160), macrophages, monocytes and DCs (ILT2/CD85j, ITL4/CD85d)[16]. HLA-G expression was originally detected in non-pathologic conditions and restricted to extravillous cytotrophoblast, thymic epithelial cells, cornea, pancreas, erythroid and endothelial precursor cells[18]. Recent studies showed that HLA-G proteins can be detected also in pathological conditions, such as in allografts and infiltrating immune cells within transplanted tissues, inflammatory diseases, virus infections and cancer[107-109]. Originally, durings the 1990s, HLA-G role in maternal-fetal tolerance preventing attack of the fetus by the maternal immune system by its interaction with uterine NK cell inhibitory receptors was demonstrated[110]. This binding through KIRDL4 receptor stimulates secretion of cytokines and angiogenic factors from NK cells, which favours implantation and placental vascularisation and development. Trophoblast itself secretes HLA-G modulating balance among these proangiogenic and antiangiogenic factors. The tolerogenic role of HLA-G in pregnancy is strongly supported by the correlations between HLA-G down regulation and preeclampsia/spontaneous abortions events[111-113]. These preliminary evidences observed in maternal-fetal tolerance suggested also that microenvironment factors may modulate HLA-G expression in tissues. Ectopic expression of HLA-G in damaged cells or tissues may be enhanced by stress, nutrient deprivation, hypoxia, hormones such as progesterone, cytokines (GM-CSF, IFNs, IL-10, TNF-α, TGF-β, LIF)[16] and immunosuppressive drugs[114].

HLA-G gene is composed of eight exons and seven introns with a stop codon at exon 6, a quite large 5'UTR extending at least 1.4 kb from ATG, and a 3'UTR[32]. The coding exons transduce only the heavy chain of the molecule and are located on chromosome 6, while β2-microglobulin (β2m) is encoded by a separated gene on chromosome 15. Exon 1 encodes the signal peptide, exons 2, 3 and 4 the extracellular α1, α2 and α3 domains respectively, and exons 5 and 6 the transmembrane and the cytoplasmic domain of the heavy chain. Exon 7 and 8 are not translated. The 3'UTR is included in the exon 8[21]. Classical HLA class I molecules are characterized by nucleotide sequence variations around the peptide-

binding cluster encoded by exon 2 (α1 domain) and exon 3 (α2 domain), while HLA-G nucleotide variability spans through exon 2, 3 and 4 (α3 domain)[21,22]. HLA-G coding sequence has a limited genetic variability in contrast to the classical HLA class I molecules that exhibit hundreds of alleles and, to date, 49 different alleles and 15 related proteins have been recognized[115]. On the other hands, 5' UTR and 3'UTR segments are high polymorphic, both influencing *HLA-G* expression modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively[19] (Figure 1).

Indeed, HLA-G may presents 7 protein isoforms generated by alternative splicing of the primary transcript. 4 isoforms are membrane-bound (HLA-G1, G2, G3 and G4) and 3 are soluble (G5, G6 and G7) species[23]. HLA-G1 and HLA-G5 are the most common isoforms observed, but HLA-G1 was the first isoform to be discovered in healthy tissues and first implicated in maternofetal tolerance[116]. HLA-G1 is the complete protein similar to the other classical membrane bound HLA-I molecules associated with β2m[23]. Crystal structure of the protein have shown that the full-length HLA-G1 is composed of a heavy chain non-covalently associated with the β2m molecule, and a peptide of about 8-10 amino acids similar to that found in the other classical I HLAs[117]. HLA-G2 isoform has no α2 domain, HLA-G3 has no both α2 and α3 domains, HLA-G4 does not present α3 domain[21]. This high post transduction availability in HLA-G molecules suggests a deeper modulation due to alternative splicing involving mostly 3'UTR region. We speculate that it could be tissue specific considering that miRNA are differentially expressed, and this modulation may be influenced by inflammation status and immune contexture microenvironment. It is known that KIR2DL4 present on NK and T cells binds HLA-G through α1 domain, or ITL-4 (DCs, monocytes and macrophages) and CD8 (T and NK cells) bind *via* α3 domain, or ITL-2 *via* α3 domain in association with β2m, however, the exact role of the less common HLA-G isoforms has not been elucidated[118]. HLA-G dimers may also be formed *via* a Cys42-Cys42 intermolecular disulfide bond on the α1 domains of the heavy chains from two HLA-G monomers. These HLA-G dimers exhibit enhanced binding avidity for ITL2/4-mediated signaling[119]. Soluble and not bound HLA-G5, HLA-G6 and HLA-G7 species have the same extraglobular domains of HLA-G1, HLA-G2 and HLA-G3 respectively[21]. It should be pointed that another form of HLA-G may be generated by proteolytic shedding of the isoform HLA-G1 (sHLA-G1)[120] and potentially anchored HLA-G2-G4, can also be shed from the cell surface if expressed. It should be highlighted that sHLA-G1 is analogue to the sHLA-G5 isoform. Soluble isoforms consequent to secretion or shedding, especially the most common sHLAG5 and sHLA-G1, can be detected in body fluids such as plasma, serum, ascites, cerebro spinal fluids exudates from patients with inflammatory diseases o cancer[17,121,122]. Similarly to membrane bound HLA-G1, sHLA-G exerts immunosuppressive functions *vs* cells of the immune contexture. Furthermore, sHLA-G produced by immune cells and/or by cancer cells induces apoptosis of activated $CD8^+$ T cells by binding to CD8 and by triggering a Fas/FasL-dependent pathway[119]. Moreover, the release of IL-3, IL-4 and IL-10 is stimulated by sHLA-G[17]. Increased sHLA-G levels compared to healthy controls have been reported in successful pregnancy after *in vitro* fertilization (IVF)[121], in patients with lymphoproliferative disorders[29], melanoma[30], and different type of cancers[23,31]. sHLA-G has been proposed as a diagnostic biomarker in CRC with increased specificity and sensibility respect to the well known carcinoembryonic antigen (CEA) protein[27]. Due to the possibility that serum sHLA-G is trapped within during the clot formation, to evaluate true biological sHLA-G levels, it is recommended to detect sHLAG in plasma[123]. Anyway, many studies still present data from serum samples.

sHLA-G levels are commonly detected with classical ELISA assay by the use of the monoclonal antibody (MoAb) MEM-G9 which recognizes (the precise epitope is unknown) the native HLA-G molecule in β2m associated forms, HLA-G1 and HLA-G5 soluble and not membrane bound isoforms[124]. It should be noted that recently, Zhao *et al*[125] demonstrated by flow cytometry that the MEM-G9 antibody it is able to bind also HLA-G3 isoform that is β2m free, thus speculating that an epitope on MEM-G/9 localized on the α1 domain of HLA-G. HLA-G may be expressed at the cell surface and also secreted. Anyway, sHLA-G can also be produced into the cells and subsequently incorporated in microvesicles, the exosomes such as in cancer[126] (Figure 2). In exosomes, HLA-G molecules form high molecular weight complexes through disulfide bridges, share partially an ubiquitinated phenotype and can be released by exosomes as demonstrated recently *in vivo*[127]. Moreover, very preliminary data coming from the HLA-G Conference held in Paris in 2012, showed that in plasma samples of healthy controls sHLA-G is preferentially exosomal-bound, while in plasma samples of lung cancer patients the level of free "not exosomal-bound" sHLA-G is increased[128]. Intriguing, it was demonstrated a mechanism of protection and immune evasion for HLA-G negative tumor cells that are in proximity of HLA-G positive tumor cells. These HLA-G expressing tumor cells by "trogocytosis" transfer membrane patches containing HLA-G molecules to active and surrounding NK cells[129]. Upon acquisition of HLA-G1-containing membranes from tumor cells, effector NK cells stop proliferating, stop being cytotoxic toward legitimate targets, and behave as regulatory cells capable of inhibiting the cytotoxic functions of other NK cells. This immediate functional inversion from an effector cell to a regulatory cell is directly due to acquired cell-surface HLA-G1[16]. We hypothesize that this mechanism could be related also to exosome sHLA-G release in the extracellular medium (Figure 2) for its re-capture, under the influence of unknown factors, may be chemokines and/or cytokines, but experimental investigations

are needed. Moreover, we hypothesize a direct role of HLA-G in affecting the IS value considering its direct inhibitory properties over NK cells and CTLs[130] (Figure 2). This topic could represent a matter of debate in the early future, not only for CRC.

HLA-G MODULATION REFLECTS 5'UTR AND 3'UTR GENETIC VARIABILITY

A growing body of evidence has been focusing in the last years on the 3'UTR polymorphisms and haplotypes, due to the microRNAs (miRNAs) interaction and their influence on expression. In particular, at least eight distinct haplotypes and eight SNPs have been described so far: the14-bp Insertion/Deletion (INDEL), +3003 T/C, +3010 C/G, +3027 C/A, +3035 C/T, +3142 C/G, +3187 A/G, and +3196 C/G[19] (Figure 1). INDEL and +3187 A/G SNPs have been associated with HLA-G mRNA stability and degradation processes even if, the exact mechanisms are not already been well elucidated[21,131]. In presence of the 14-bp Insertion (5'-ATTTGTTCATGCCT-3') sequence, HLA-G alleles have been associated with lower mRNA production[132]. In the other hand, it has been demonstrated that a smaller fraction of HLA-G mRNA transcripts presenting the 14-bp Insertion (Ins) can be alternative spliced from the mature HLA-G with the removal of 92 bases of exon 8 (include SNPs +3003 and +3010)[19]. mRNA producing smaller mRNA transcripts, reported to be more stable than the complete forms[20]. Interestingly, some AU-rich elements (ARE) are present in the 3'UTR of HLA-G, and it is known that these sequences are recognized by proteins causing rapid changes in mRNA stability[19,32]. The 14-bp sequence begins with AUUUG, and the absence of such motif in the 92 base-deleted transcripts might give an explanation of their resistance to degradation processes[20]. The presence of a guanine in the position +3142 increases the affinity of specific miRNAs (miR-148a, miR-148b and miR-152) to the HLA-G mRNA, decreasing HLA-G expression[32,133] (Figure 1). The influence of +3142G allele was demonstrated by functional studies in which HLA-G high expressing JEG-3 cells were transfected with miR-148a: decreased soluble HLA-G levels were detected[134].

In Table 1 are listed the associations found among HLA-G UTR SNPs and alleles, in particular the 14 bp INDEL polymorphism, and various disorders including cancer. Associations with circulating HLA-G levels were also reported if investigated. Recently, the risk of invasive cancer of uterine cervix was found to be significantly increased in presence of the 14 bp I/I and also the HLA-G*01:01:01:02 genotype in a large Canadian study of 539 women with histologically confirmed HG-CIN and invasive cancer; 833 women with normal cytology served as controls[33]. Moreover, 14 bp I/I genotype correlates with disease progression from high-grade cervical intraepithelial neoplasia (CIN3) to invasive cancer[33]. Previous data provided evidences of *HLA-G* expression in association with tumor metastasis and poor survival in an ovarian cancer animal model[135], and also with NK cell cytotoxicity inhibition and MMP-15 expression in ovarian cancer cell line[136]. In Kazakh population the risk of developing esophageal carcinoma was significantly higher in individuals carrying the 14 bp Del/Del (D/D) genotype[34], while Teixeira *et al*[35] showed that the 14 bp (D/D) genotype increases hepatocellular carcinoma (HCC) susceptibility in Brazilian population. Conversely, the 14 bp INDEL was no associated with HCC and liver cirrhosis susceptibility in a Korean study[137]. The 14 bp I/I was also found to be related to lower OS and DFS in patients with haematological malignancies undergoing allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) and Methotrexate (MTX) treatment in univariate and multivariate analysis[138]. The 14 bp I/I genotype was also suggested to represent a therapy marker in Rheumatoid Arthritis (RA), able to identify responder patients treated with MTX[114].

To date, an association among the +3142 C>G SNP and a specific disease status, was recently described only in the Systemic Lupus Erythematous (SLE) autoimmune disorder[37] and in Relapsing-Remitting Multiple Sclerosis (RR-MS)[139] (Table 1).

A first attempt to find possible correlations between HLA-G genotype and phenotype (sHLA-G) was performed by Rebmann *et al*[140] in 2001, analyzing 94 healthy subjects. In particular, individuals carrying the HLA-G*01041 allele had significantly higher sHLA-G levels, while individuals with HLA-G*01031 allele and HLA-G*0105N allele, presented significantly lower of plasmatic and circulating HLA-G[140]. It should be noted that the 5'UTR and 3'UTR HLA-G regions were not investigated. The 14 bp I/I was then associated with significantly lower levels of sHLA-G in blood plasma or serum in different studies[141,142] (Table 1). Chen *et al*[143] reported a dramatic and significantly lower expression of sHLA-G in plasmatic samples from healthy donors (n = 150) of Chinese etnicity in the presence of the 14 bp I/I genotype with respect to the 14 bp D/D. Another recent investigation performed in MS patients in serum and cerebro spinal fluid (CSF), demonstrated an association among higher sHLA-G levels and +3142 C/C, 14 bp D/D genotypes and lower sHLA-G levels in +3142 G/G, 14 bp I/I combination[139] (Table 1).

Rizzo *et al*[144] identified a subgroup of Early Rheumatoid Arthritis (ERA) patients characterized by prevalence in 14 bp D/D, 14 bp D/I polymorphisms, and improved disease remission, therefore highlighting a protective role for the 14 bp Del allele. Moreover, the 14 bp Del allele was associated with higher sHLA-G and mHLA-G production and ITL2 expression. In 2010 Castelli *et al*[19] defined almost eight distinct 3'UTR haplotypes named from UTR-1 to UTR-8, that include the eight common polymorphisms of this nucleotide segment described above. Furthermore, HLA-G alleles were associated with each haplotype, and high Linkage Disequilibrium (LD) among most of the variants was observed, according to Hardy-Weimberg test. In particular, it was evidenced that the 14

bp Ins allele is always associated with the +3142G and +3187A alleles, both previously related with low mRNA availability, thus suggesting the implication of these two polymorphisms in lower mRNA production is associated with 14 bp insertion (Figure 1). The+3187G allele is only associated with the 3'UTR-1 haplotype carrying the 14 bp deletion[19].

It should be emphasized that 4-bp upstream the SNP +3187A/G there is an AUUUA motif, and 9-bp downstream to +3196C/G there is the presence of an UUAUUU motif. These (AU)-rich elements may modulate mRNA degradation and therefore expression level, and are also influenced by close sequence variations. Some authors, according to this nomenclature, started to analyze data from 3'UTR region in terms of haplotypes using different algorithm approaches such as EM algorithm, PHASE method, and FBAT. This haplotype analysis could represent an amazing way to correlate genetic data to the specific phenotypes of the study grouped also in a dominant or in a recessive model, or used to compare a single common haplotype with other phenotypes grouped together. To date, 3'UTR haplotypes analyses were performed in few studies and not regarded to cancer disease and so to CRC[113,131,145].

Recently, the functional impact of 3'UTR and in particular the 14 bp INDEL polymorphism, was deeply investigated by Svendsen *et al*[146], with particular attention on the processing and stability of the full-length membrane bound transcript of HLA-G1 (mHLA-G1). Authors, transducing different HLA-G1 DNA sequences in K562 human cell line, demonstrated that mRNA from 14 bp I/I had a higher degree of stability than the others, in accordance to the data reported in literature. Moreover, transductants carrying the 14 bp Ins, presented lower sHLA-G1 levels per mHLA-G1 ratio with respect to the constructs lacking the 14 bp Ins, but were the most efficient in inhibiting NK cytotoxicity[146].

In regard to the 5'UTR region, it has been less investigated and only recently, 16 SNPs in the 5'UTR region (Figure 1) and the 14-bp INDEL polymorphism in 3'UTR were analyzed in the same study in Brazilian patients who underwent assisted reproduction treatments (ART), characterized by failure implantation of embryos[113]. Larsen and Hviid[133], presented a complex panel of haplotypes related to the 5'UTR and in part of the 3'UTR regions showing clear LD between several of the polymorphisms centered around two main lineages of HLA-G alleles named G*010101$_{xx}$ and G*010102$_{xx}$, in accord to WHO classification. The HLA-G promoter region is considered unique among the *HLA* genes with many regulatory sequences, such as tissue specific regulatory element (TSRE) from position -1350 to -1100, and IFN-stimulated response element (ISRE) from -726 to -725 position[21,147]. The 5'UTR tri-allelic polymorphism -725C/G/T was evaluated in relation to plasma sHLAG concentration in a recent study in Iraqi women with recurrent pregnancy loss[148] A significantly association among lower levels of sHLAG in presence of the CC genotype was found *vs* the CG and CT condition[148] (Table 1). Of note, the presence in this position of a G nucleotide may alter the methylation profile of CpG di-nucleotides resulting in a modification of gene expression[113], and also influences binding of ISRE or other regulatory elements.

HLA-G IN CRC AND FUTURE APPLICATIONS

Despite the advances in knowledge and increasing interest in the immune contexture involvement, HLA-G has been poorly investigated in CRC and inflammation associated diseases of the gastrointestinal tract. A common mechanism present in CRC and in various types of cancer used by tumor cells to avoid recognition by CTLs, is the HLA class I down-regulation or total loss[149]. Altered HLA I class phenotypes regard reversible down regulation or irreversible mutational genes inactivation and are usually related to LOH of classical HLA-A, -B and -C heavy chains located in the chromosome region 6p21. HLA-G (protein and/or mRNA) is frequently over-expressed in tumors[16,17], including CRC[16,17,24-26] (Table 2). HLA-G expression was associated with malignant transformation and was never detected in the surrounding and closest areas near the tumor[17]. MHC class I loss or down-regulation due probably in defects or alterations in Processing Machinery (APM) components have been found in different malignancies and associated to reduced MHC class I recognition *vs* tumor-associated antigen (TAA)-specific CTL and disease progression[150]. LOH in the 15q21 region was observed in progressing lesions after immunotheraphy such as in melanoma[151]. Intriguingly, while classical HLA I proteins are frequently down regulate in in about 15% up to 75% of colon carcinoma lesions[150,152,153], HLA-G (related to isoform G1) results over-expressed in CRC malignant and pre-malignant tissues[26] (Table 2), in accord to his role in the host immune escape. Strong positive HLA-G expression was also detected in UD biopsies but no in tissues taken from patients affected by CD, thus it was proposed as a tool to better distinguish these inflammatory diseases[154] (Table 2).

LOH frequency for classical *HLA* genes in CRC was reported to be 40% evaluating 95 patients[155]. In CRC, higher LOH percentages were found in other chromosomal regions containing tumor suppressor genes *i.e.*, 43%-79% at 18q, 43%-76% at 17p and 17%-43% at 5q[156]. The irreversible total loss of HLA class I is generally referred to mutations affecting the β2m gene that are usually followed to loss of the second copy by LOH within his locus in the 15q21 region[157,158]. Expression of the β2m protein should be taken in consideration due its fundamental role in associate to HLAs molecules for correct antigen presentation. If β2m is lost, stable antigen-HLA class I complexes cannot be produced[156]. Of note, the major function of HLA-G is not antigen presentation and, if β2m is necessary to assemble the most studied HLA-G1, sHLA-G1 and sHLA-G5 isoforms, it should

be discussed the function of alternative spliced isoforms lacking β2m. We speculate that HLA-G alternative spliced isoforms without β2m assembly could represent another way to escape from immune control, especially in tumor such as CRC in which β2m downregulation or loss is a frequent event[158]. Moreover, their role should be established and explored (which are their interactors?) providing new insides of the HLA-G planet.

Alterations due to LOH have been reported in 25%[159]-35%[156] of CRCs in β2m 15q21 and in 6p21 in 40% of the same patients[156], demonstrating a strong correlation that may impact on disease progression in terms of immune escape exert by the tumor[160]. To date in CRC, HLA-G polymorphisms and haplotypes have not been investigated to find correlations with prognosis or phenotype (circulating sHLA-G proteins) even if, recently, some authors started to report complex and intriguing analysis not related to cancer[147,148].

Data about the soluble HLA-G has been reported only for CRC patients of Chinese ethnicity[27,28] suggesting that sHLA-G should be considered as a good diagnostic tool superior to classical CEA[27], and also a useful indicator to distinguish benign colorectal related disease from CRC. Of note, Cao *et al*[28] collected and analyzed plasma samples from a limited patient series, while Zhu *et al*[27] quantified sHLA-G in a quite large cohort of patients in serum that is not the recommended biological sample (Table 2). Both authors did not genotype patients at the germinal level to search for a possible phenotype correlation and/or to assess specific HLA-G alleles and genotypes related to CRC. No correlations were performed with clinical outcome of CRC patients in terms of survival, disease relapse and response to therapy treatment. In our opinion, detection of sHLA-G is not sufficient alone and should be associated to genotyping of the gene, with particular attention on the regulatory untranslated regions that are susceptible to post translational modifications such as methylation, transcription factors and miRNA binding (Figure 1). Moreover, sHLA-G assay should be standardized and defined by precise guidelines to improve its clinical application. Recently, preliminary data that need further investigations, showed that higher sHLA-G expression in mucor secretory *vs* non-mucosecretory CRC, correlate with worse prognosis of patients[128].

A differential spontaneous sHLA-G secretion from PBMC was reported in CD (high levels of the secrete molecules) and a lack of sHLA-G in UC also after inflammatory stimulus by Lipopolysaccharide (LPS) activation[161] (Table 2). Conversely, opposite results were previously described analyzing HLA-G protein expression by IHC in CD and UC biopsies, with higher expression levels in UC and negative staining in CD samples[154] (Table 2). Subsequently, these controversial data were not confirmed with novel studies. However, authors who reported a lack of sHLA-G in UC[162], showed that immunosuppressive drugs influence the sHLA-G secretion in UC and CD patients, stimulating its release in UC and decrease it in CD[162] (Table 2). Halama *et al*[163], basing on the evidence that CRC patient prognosis is dependent on the local immune contexture, characterized NK and T cells localization and densities in primary CRC liver metastasis, adenomas and normal tissues. NK cells were rare in tumor tissue independently of HLA class I expression, and also not depending from chemokine levels that were rather elevated and correlated to T cells infiltration[163] Subsequently, for the first time Rocca *et al*[164], characterized tumor-associated NK cells (TANKs), with respect to autologous peripheral blood NK cells (PB-NKs), from CRC patients, and compared the latter with PB cells from healthy donors. Authors demonstrated an altered phenotype for TANKs in CRC patients, with a low expression of activating receptors and also with an impaired degranulation and release of cytokines (IFN-γ) capacity. It should be pointed that HLA-G evaluation would be of interest in both these analysis, especially considering recent data about sHLA-G role in impair NK cells by (1) modulation of specific chemokine secretion (CXCR3, CX3CR1, and CCR2); (2) functional inhibition on CD94/NKG2A receptor; and (3) modulation of NK chemokines and cytokines secretion[165].

Finally, the host-immune reaction could be the critical element in determining response to therapy, and the effect on the immune response could be the underlying factor behind many of the predictive markers[13]. In ovarian cancer, positive HLA-G cells from peritoneal and pleural effusions decrease in number after chemotherapy and this result correlates with improved survival of the related patients[166]. These data suggest that HLA-G-expressing cells are more susceptible to elimination by the immune response or treatment[162,166].

On the opposite, IFN-α immunotherapy showed to further increase circulating sHLA-G levels in melanoma patients[30], thus in accord to the presence of ISRE element in the 5'UTR region[21]. It is of knowledge that common therapies in cancer (chemotherapy and radiotherapy) have an impact on immune system[54], that could be different depending from the therapy regiment, often associated to different grades of toxicity and neutropenia[167]. A favourable prognosis correlated with TILs in stage Ⅲ CRC patients treated by surgery alone (n = 851) or 5-fluorouracil (5-FU) adjuvant chemotherapy (n = 305), was observed[168]. Moreover, high densities TILs in metastatic liver lesions at the invasive margin revealed strong association with chemotherapy efficacy and prognosis in advanced CRC patients[169,170]. These studies based on infiltrating lymphocytes in correlation with therapy, have provided the scientific basis to implement the concept of IS and, in the future, we hope that IS validation will provide a well defined tool to assess CRC prognosis and clinical outcome based also on patient treatment. Due to HLA-G functional properties in the direct inhibition of cytotoxic and memory T lymphocytes, we stress the possible association between IS and HLA-G (Figure 2) and therefore patient prognosis.

Moreover, particular HLA-G haplotypes and/or genotypes and allelic variants could be identified as predictive

genetic marker of response treatment, to better stratify patients.

In particular, further investigations in large cohort of patients are needed to define: HLA-G haplotypes, genotypes, and alleles frequencies and association with: (1) the risk of CRC (possible distinctions comparing them with those of UD and CD diseases); (2) the prognostic power: relation with OS, DFS, and predictive value of response to therapy; (3) plasma sHLA-G levels as predictive and prognostic markers; and (4) genotype-phenotype correlations among soluble and HLA-G genotypes/alleles (especially 5'UTR and 3'UTR nucleotide variations).

We suggest that HLA-G could represent a novel prognostic immune biomarker with a prominent role in inhibiting host immune response in CRC. We also suggest to assess an association with IS and HLA-G (Figure 2) to better characterize host immune response and complete the immune contexture overview in CRC patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Role of preoperative CT colonography in patients with colorectal cancer

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Abstract

In patients with colorectal cancer (CRC), accurate preoperative evaluation is essential for a correct therapeutic plan. Colonoscopy and intravenous contrast-enhanced computed tomography (CT) are currently recommended in the preoperative work-up for CRC. Preoperative colonoscopy has some limitations such as misdiagnosis of synchronous cancers in cases of incomplete exploration of the colon and inaccurate tumor localization. Intravenous contrast-enhanced CT successfully documents distant metastases although it sometimes enables unsatisfactory locoregional staging. Computed tomography colonography (CTC) is obtained after gas insufflation of the colon and offers a comprehensive preoperative evaluation in patients with CRC, including a definition of the segmental location of the tumor, presence of synchronous lesions or lack thereof, and fairly accurate locoregional staging. CTC has some limitations, including a lack of biopsy capability, suboptimal sensitivity for synchronous small polyps, and unsatisfactory nodal staging. Bearing in mind these limitations, CTC could be employed as a "one-stop-shop" examination for preoperative assessment in patients with CRC.

Key words: Colorectal cancer; Colonoscopy, Computed tomography colonography; Synchronous cancer; Cancer staging

Core tip: Computed tomography colonography (CTC) can be employed as a "one-stop-shop" examination for preoperative assessment in patients with colorectal cancer (CRC). CTC is well accepted and tolerated by patients and also accurate in the detection of significant colorectal lesions. In patients with CRC, CTC defines the segmental location of the tumor and the presence of synchronous lesions or lack thereof and provides fairly accurate locoregional staging.

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INTRODUCTION

In Europe, colorectal cancer (CRC) is the second most frequent malignant neoplasia and the second most common cause of death from cancer[1]. Whereas in the United States, CRC accounts for the fourth highest incidence of cancer and the fourth leading cause of cancer-related deaths[2].

In European countries, the average relative five-year survival rate for patients with CRC is 54%[3]. However, patient prognosis and treatment largely depend on the

Table 1 Computed tomography colonography scanning technique for a 64-slice scanner[1]

	Preoperative CTC	Screening CTC
IV contrast media	Yes	No
Patient position	Prone (unenhanced), supine (portal phase)	Supine, prone
Collimation	32 mm × 0.6 mm	32 mm × 0.6 mm
Tube voltage	120 kV	120 kV
Tube current	140 eff mAs (unenhanced), 200 eff mAs (enhanced)	50 eff mAs
Tube rotation time	0.5 s	0.5 s
Pitch	1.4	1.4
Section width	1 mm	1 mm
Reconstruction increment	1 mm	1 mm

[1]Scan parameters for Sensation 64 (Siemens, Erlangen, Germany). CTC: Computed tomography colonography.

disease stage at initial diagnosis. Accurate preoperative evaluation is essential for a correct therapeutic plan, including surgery (open or laparoscopic), radiotherapy or chemotherapy. In particular, a preoperative work-up is aimed to exclude the presence of synchronous cancers, to evaluate local invasion, and to detect nodal and distant metastases. Moreover, precise localization of the tumor is essential for surgical treatment planning, especially in the case of the laparoscopic approach.

A wide range of diagnostic tools is available to study patients with CRC, including optical colonoscopy, double contrast barium enema (DCBE), ultrasound (US), computed tomography (CT), computed tomography colonography (CTC), magnetic resonance (MR) and positron emission tomography (PET)[3,4]. CTC potentially represents a comprehensive examination for preoperative evaluation of patients with CRC. In particular, it is accurate in the detection of significant colorectal lesions[5-7]; enables evaluation of the entire colon, even in cases of obstructive lesions; and allows segmental localization of the tumor. At the same time, CTC permits staging of extra-colonic tumor spread, both locoregional and distant.

CRC may present with non-specific symptoms or signs (rectal bleeding, change in bowel habits, abdominal pain or anemia) or with acute bowel obstruction. Moreover, CRC may be discovered in asymptomatic subjects as the result of screening with fecal occult blood test, sigmoidoscopy, colonoscopy, or CTC. In all cases an ultimate diagnosis is generally made by colonoscopy and biopsy.

Herein, we shall review the technique, benefits and limitations of CTC as a preoperative examination in patients with already diagnosed CRC.

CTC TECHNIQUE

A state-of-the-art CTC examination requires adequate bowel preparation, optimal colonic distension and proper scanning technique[8]. Moreover, in patients with diagnosed CRC, CTC must be performed with an administration of intravenous-iodinated contrast media, as it allows extra-colonic organ evaluation, which is requested in search of distant metastases[8].

Bowel preparation for CTC in patients with known CRC is usually obtained with a three-day low fiber diet and the administration of a cathartic agent such as a polyetilenglycole solution the day before the CTC examination. In frail or elderly patients, a reduced cathartic preparation should be considered such as a three-day low fiber diet and the administration of 13.8 g of macrogol 3350 (Movicol; Norgine, Milan, Italy) diluted in a glass of water and given at the three main meals for three days before the examination[9]. Fecal tagging should be routinely used, as it improves colonic lesions detection without noticeably affecting image quality after administration of intravenous contrast media[8]. Fecal tagging is usually obtained with 50 mL of iodinated oral contrast agent (Gastrografin; Bayer Schering Pharma AG, Berlin, Germany) administered 2-3 h before the procedure.

Colonic distension should preferably be performed using an automatic carbon dioxide insufflator[10], although the manual insufflation of room air is acceptable. Before insufflation, if there are no contraindications, including hypersensitivity to the active principle, untreated narrow angle glaucoma and prostatic hypertrophy with urinary retention, we intravenously administer 20 mg of scopolamine butylbromide (Buscopan; Boehringer Ingelheim Italia, Milan, Italy) to improve colonic distension[11]. In patients with stenosing lesions, insufflation should be gradually performed and carefully monitored using CT scout views, as the risk of perforation, although extremely low for CTC, could be increased[12].

The recommended scanning technique for preoperative CTC differs from that adopted for screening CTC, including patient positioning, scan parameters concerning the delivered radiation dose and administration of intravenous contrast media (Table 1)[13]. In our institution, for the preoperative evaluation of CRC, a preliminary unenhanced acquisition is performed in a prone position. Then, a portal phase supine scan is performed 70 s after the administration of contrast media[8]. The use of a multi-phasic CT protocol after administering contrast media may be chosen by the radiologist in specific clinical settings (*e.g.*, patients with non-characterized liver focal lesions). An arterial phase thoracic acquisition can also be performed as part of staging, when appropriate. All images are then transferred to a dedicated workstation, which allows visualization of two-dimensional axial and multi-planar reformatted (MPR) images, three-

dimensional endoluminal surface-shaded images (SSD) and double-contrast-like reconstructions of the colon.

DIAGNOSIS OF SYNCHRONOUS CANCERS

An important issue regarding patients with CRC is the occurrence of synchronous cancers (SC), which are reported in 2%-11% of the cases[14-16]. A search for SC is routinely performed during open surgery for CRC, but intra-operative palpation of the colon can miss up to 69% of SC[17]. Moreover, in case of the laparoscopic approach to CRC, the surgeon cannot explore the entire colon in a search for simultaneous lesions. Missed diagnosis of SC can lead to increased morbidity and progression of CRC to a more advanced stage[18]. In fact, preoperative identification of SC implied a more extended colonic resection in 11%-44% of cases[18]. Because patients undergoing preoperative conventional colonoscopy have fewer local recurrences, fewer distant metastases and a longer disease-free survival time[19], full preoperative colonic evaluation with colonoscopy should always be performed in patients with CRC.

In case of incomplete colonoscopy due to an insuperable stenosing cancer or other causes (*e.g.*, inadequate bowel preparation, anatomic variants, fixed colon segments, patient's intolerance to the procedure), endoscopic diagnosis of synchronous lesions may be precluded. A recent study showed that advanced neoplasia could be missed in up to 4.3% of patients during incomplete colonoscopy, suggesting that further colonic evaluation is mandatory in these cases[20]. To complete evaluation of the colon, radiological examinations can be performed, such as double-contrast barium enema (DCBE) and CTC.

A multi-centric randomized trial comparing diagnostic performance of DCBE and CTC in patients with suspected CRC clearly showed that diagnostic accuracy of DCBE for CRC is not satisfactory[21]. In particular, barium enema missed 12 of 85 cancers[21]. However, CTC showed a sensitivity for cancer and adenomas larger than 10 mm comparable to colonoscopy, namely 96.1%[5] and 84%-92.2%[6,7]. Moreover, CTC is well tolerated by patients[22], and its complications are exceedingly rare[12]. However, CTC has some limitations. It does not allow the biopsy or removal of discovered lesions, precluding histological diagnosis. Moreover, its sensitivity for intermediate polyps (6-9 mm) is lower than that of colonoscopy, namely 70%, and even worse, 48%, for diminutive lesions (< 5 mm)[23]. In fact, small polyps can be overlooked by preoperative CTC.

Several studies showed that CTC represents a valuable tool to evaluate the proximal colon after incomplete colonoscopy[24-28], and the American Gastroenterologists Association (AGA) recognized that CTC is indicated for adults with failed colonoscopy[29]. Several studies evaluated the role of CTC in patients with CRC and incomplete colonoscopy. In patients with CRC, a CTC with complete colonic distension was achieved in 83% to 100% of the cases[24,30-32]. In a series of 174 patients with CRC, McArthur *et al*[32] showed that all synchronous cancers and 83.3% of synchronous polyps greater or equal to 10 mm were identified by CTC. Other studies with smaller groups of patients reported that CTC depicted all synchronous cancers and had a sensitivity for polyps greater or equal to 10 mm of 100%[24,30,31].

One study showed that CTC is technically feasible and well tolerated also in patients with CRC presenting with acute or subacute bowel obstruction[33]. Finally, a study proved that CTC is a safe and useful method for preoperative examination of the proximal colon after metallic stent placement in patients with acute colon obstruction caused by cancer[34].

Overall, the above data indicate that CTC, despite its limitations, should be the examination of choice to complete colonic examination after incomplete colonoscopy in patients with CRC (Figure 1), as it reliably detects synchronous cancers and polyps, allowing surgical removal of cancer and/or post-operative endoscopic polypectomy.

TUMOR LOCALIZATION

Tumor localization is another significant issue of preoperative work-up for CRC, especially in the case of the laparoscopic approach. Laparoscopic surgery for CRC is increasing in clinical practice as it showed comparable results to those of open surgery in randomized trials[35]. Accurate preoperative localization is fundamental in laparoscopy-assisted colectomy because the colon cannot be palpated during the procedure and the lesion may not be apparent on the serosal surface, adding the risk of removing the wrong colonic segment[36].

Precise endoscopic localization of the tumor can be challenging as anatomical landmarks may not be readily apparent at colonoscopy and often only the distance from the anal verge is recorded. The endoscopist can also be confounded by the presence of a redundant colon or anatomic variants. In fact, studies showed that colonoscopy has a suboptimal accuracy in locating the tumor, which can be incorrect in 14%-21% of the cases[37,38], especially in the sigmoid and descending colon[37].

Other techniques can be used to precisely localize colonic lesions, such as barium enema, CTC, endoscopic tattooing, and intraoperative colonoscopy[39].

As DCBE is a suboptimal tool to detect CRC, its usefulness for tumor localization is questionable. Moreover, DCBE does not clearly show the position of the various colonic segments in the three dimensions leading to potential errors, especially in the transverse and sigmoid colon. CTC clearly demonstrates the involved colonic segment, the length of tumor extension and its relationship with adjacent organs, vascular structures and peritoneal spaces. On 94 patients with CRC, the accuracy of CTC for tumor localization was 94.7%[39]. In 65 patients with CRC, the sensitivity and specificity of CTC in

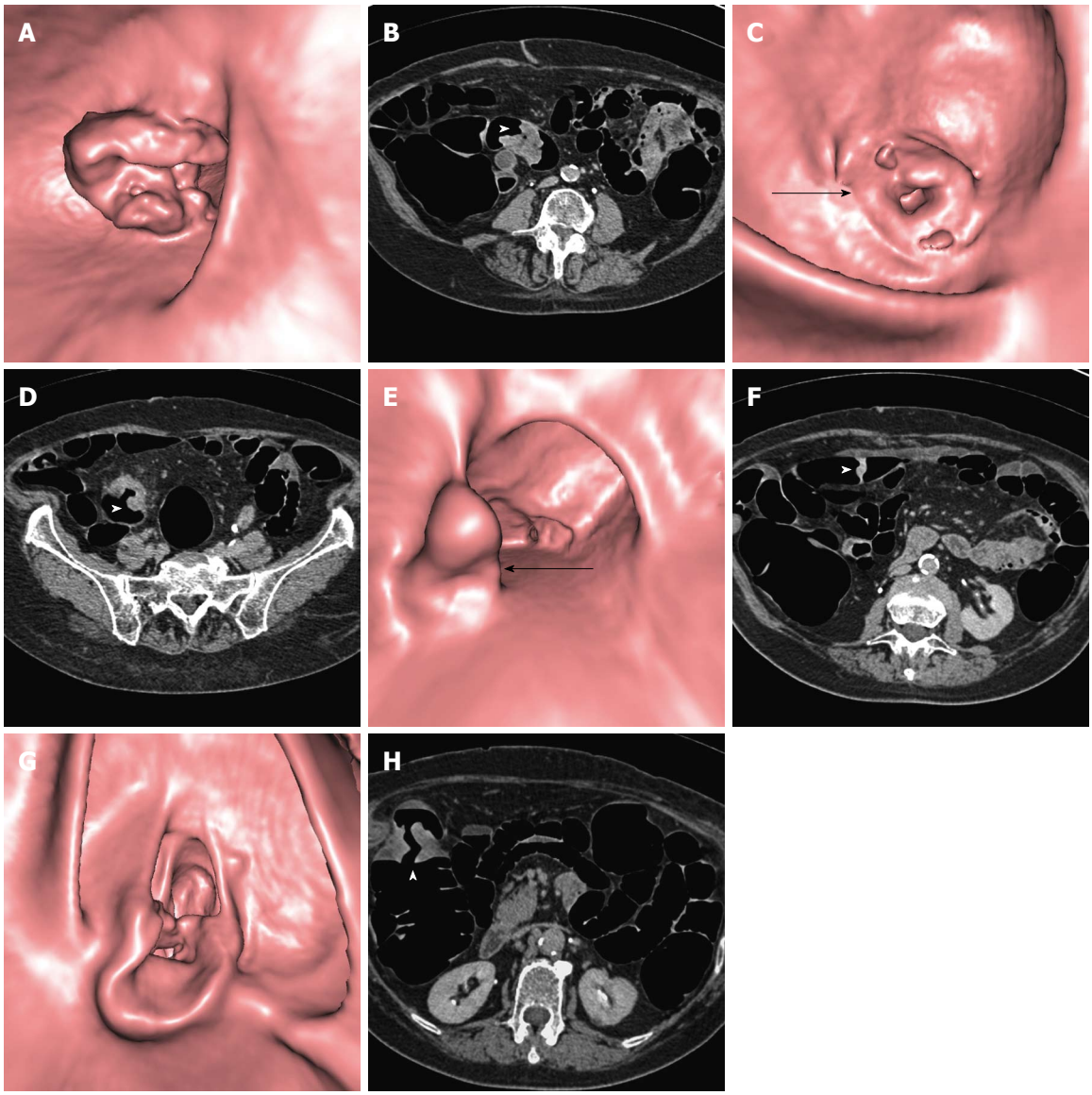

Figure 1 Computed tomography colonography performed in an 83-year-old female with incomplete colonoscopy due to stenosing adenocarcinoma of the sigmoid colon. Computed tomography colonography (CTC) identified three other synchronous colonic tumors confirmed as adenocarcinomas at surgery. CTC endoluminal and axial source images are shown. A, B: Distal stenosing lesion of the sigmoid (arrowhead); C, D: Proximal stenosing lesion of the sigmoid (arrow and arrowhead); E, F: Vegetating lesion of the transverse colon (arrow and arrowhead); G, H: Stenosing lesion of the right flexure (arrowhead).

determining the location of colonic masses were found to be 100% and 96%, respectively, whereas colonoscopy failed to precisely localize the tumor in 24% of cases[40].

CANCER STAGING

Treatment of CRC depends on the preoperative assessment of disease extension. Colorectal carcinomas are clinically staged using the TNM system established by the American Joint Committee on Cancer (Table 2)[41]. Chest and abdominal CT is recommended for the preoperative staging of CRC by the European Society of Medical Oncology[3] and by the American College of Radiology[4], with the exception of rectal cancer, for which magnetic resonance is more accurate for T staging. Although CT is the examination of choice, disappointing results have been reported with staging accuracy, ranging from 48% to 77%[42]. CTC potentially represents a comprehensive examination for CRC staging, as it allows for the evaluation of the inner and outer colonic wall (T stage), pericolonic lymph nodes (N) and distant metastases (M) (Figure 2).

Several studies evaluated accuracy of CTC for T and N staging of CRC[42-47]. Because CT cannot discriminate the different bowel wall layers, a simplified T staging system has been proposed for CTC reports with a grouped T1/T2 category for lesions confined to the bowel wall, T3 category for lesions invading subserosal fat and T4 category for cancer invading adjacent organs[42].

To distinguish T1/T2 from T3 cancers, both colonic wall deformity and lesion outer borders should be considered. As suggested by Utano *et al*[44], intestinal wall

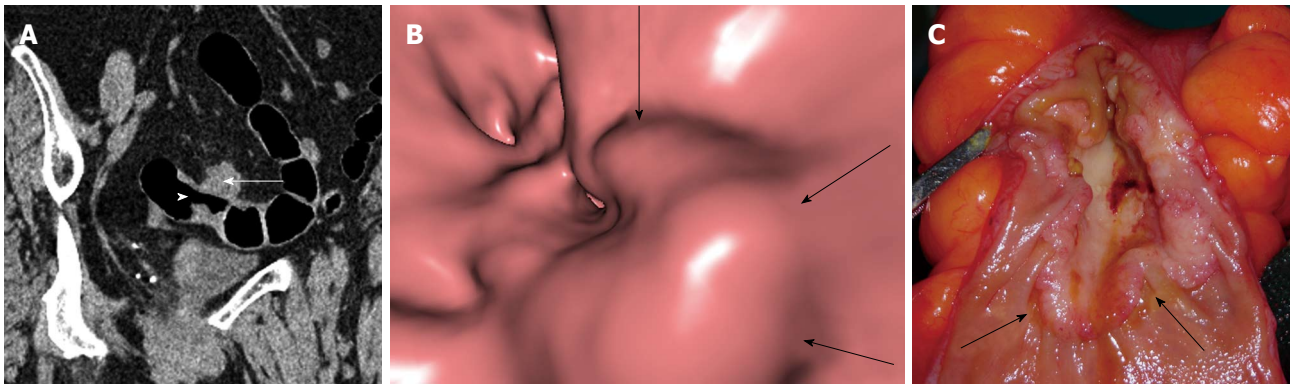

Figure 2 Stenosing adenocarcinoma of the sigmoid colon. A: A Computed tomography colonography (CTC) oblique coronal reconstructed image depicts the lesion (arrowhead) and its nodular infiltrating margins in pericolonic fat (arrow); B: An endoluminal CTC image better shows lobulated inner borders (arrows) of the lesion; C: A surgical specimen from left hemicolectomy shows the stenosing lesion with its lobulated inner borders (arrows).

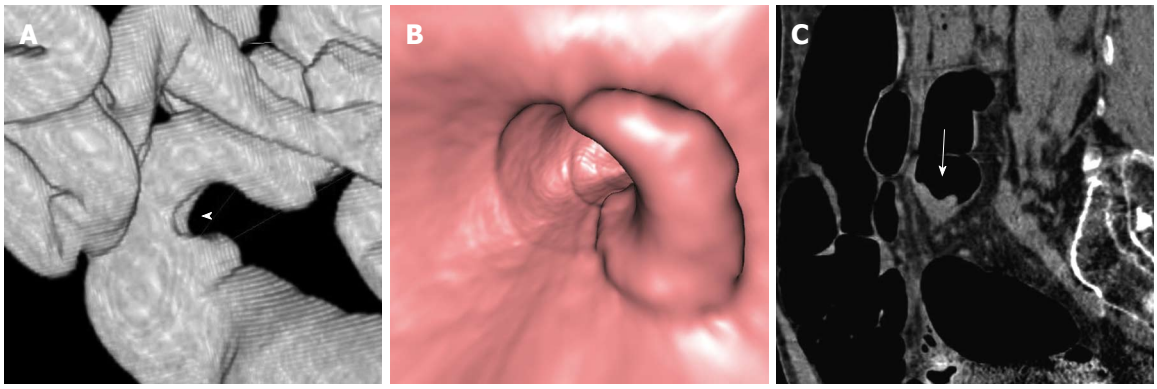

Figure 3 Computed tomography colonography of a T2 vegetating lesion of the sigmoid colon. A: A Computed tomography colonography (CTC) SSD reconstructed image shows a trapezoid type wall deformity of the sigmoid colon (arrowhead); B: A CTC endoluminal image demonstrates that wall involvement is less than 50%of luminal circumference. C: A CTC sagittal reconstructed image shows that the lesion (arrow) has sharp margins.

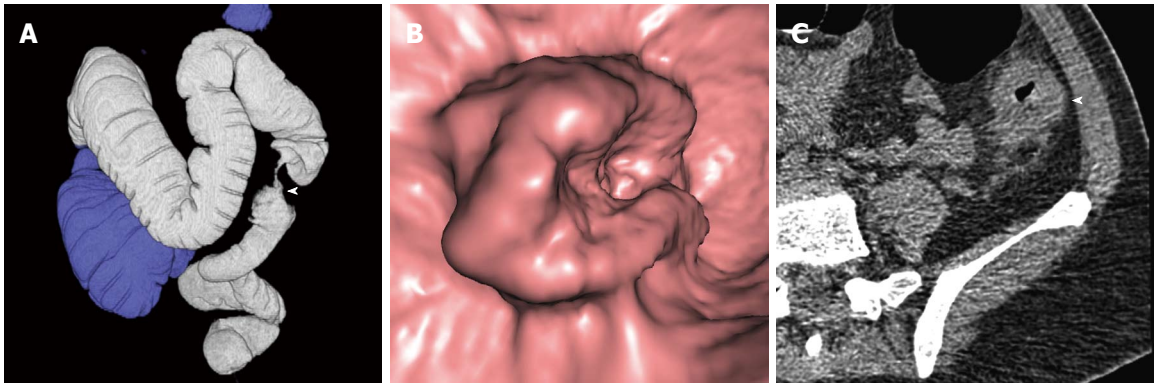

Figure 4 Computed tomography colonography of a T3 stenosing lesion of the descending colon. A: A Computed tomography colonography (CTC) SSD reconstructed image shows an apple-core type wall deformity of the descending colon (arrowhead); B: A CTC endoluminal image demonstrates that wall involvement is more than 50% of the luminal circumference; C: A CTC axial source image shows that the lesion (arrowhead) has nodular infiltrating margins.

deformity associated with CRC and observed on SSD reconstructions can be classified into arc type, trapezoid type, and apple-core type. Arc type is defined as a smooth concave wall deformity, trapezoid type is defined as a square-like irregular wall deformity involving less than 50% of the circumference of the lumen, and apple-core type is defined as a trapezoidal wall deformity involving more than 50% of the circumference of the lumen. The arc or trapezoid type is associated with T1/T2 cancers (Figure 3), whereas the apple-core type is associated with T3/T4 (Figure 4). In a series of 246 patients with CRC, the sole evaluation of wall shape deformity, as described above, showed an overall accuracy for T staging of 79%[44]. With a similar classification of

Table 2 TNM staging of colorectal cancer[1]

Stage	Description
Primary tumor (T)	
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into the subserosa, or into the non-peritonealized pericolic tissues
T4	Tumor directly invades other organs or structures and/or perforates the visceral peritoneum
Regional lymph nodes (N)	
N0	No regional lymph node metastases
N1	Metastases in 1-3 regional lymph nodes
N2	Metastases in ⩾ 4 regional lymph nodes
Distant metastases (M)	
M0	No distant metastases
M1	Distant metastases

[1]American Joint Committee on Cancer (AJCC).

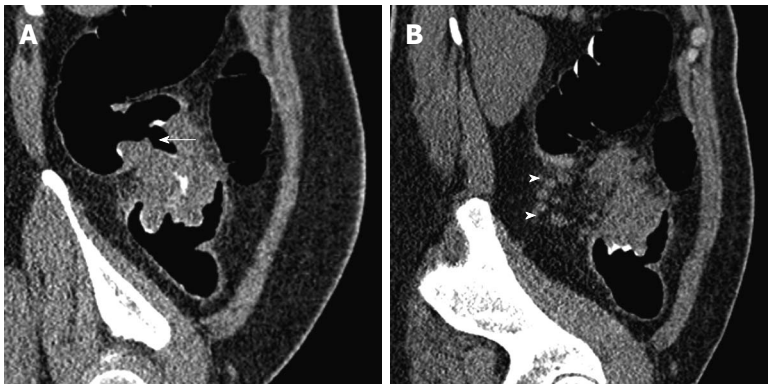

Figure 5 Computed tomography colonography of a T3 N2 stenosing computed tomography colonography of the descending colon. A: A Computed tomography colonography (CTC) sagittal reconstructed image shows the lesion (arrow); B: A CTC sagittal reconstructed image demonstrates four subcentimetric perivisceral lymph nodes (arrowheads).

wall deformity, an accuracy of 77.6% was reported in another study[43].

Colonic wall outer margins should also be evaluated to distinguish T1/T2 from T3 cancers. Rounded or nodular advancing margins in perivisceral fat are considered an expression of a T3 stage cancer[42]. The presence of spiculations within the fat is not universally considered a sign of pericolonic fat invasion, as spiculations can be caused by inflammatory reactions and extramural fibrosis[48]. Direct invasion or absence of a fat cleavage plane from an adjacent organ indicates a T4 stage cancer. Using the above-mentioned criteria, the overall accuracy of CTC for T staging ranged from 66% to 95%[42-47].

The identification of nodal involvement with CT is limited by the use of dimensional and other morphological criteria, such as clustering. In particular, as proposed by Filippone *et al*[42], N1 stage can be assumed on CTC if a cluster of three nodes is present, independent of their size, or if fewer than three lymph nodes are present, with at least one of the nodes measuring 10 mm or more in the long axis. In stage N2 neoplasms, more than three perivisceral lymph nodes are identified, regardless of their size (Figure 5). Using these criteria, the overall accuracy of CTC for N staging ranged from 70% to 85%[42-47]. Notably, the accuracy of CTC for nodal staging may be unsatisfactory because the presence of regional lymph-node metastases represents an important indication for adjuvant chemotherapy, and up to 30% of node-negative patients eventually develop distant metastases, possibly as a consequence of lymph-node micrometastases[49].

It has been emphasized that the use of MPR images[45], and in particular of true axial images along the short axis of the colonic segment[50], improves the depiction of the outer margins of the lesion, of the relationships with adjacent organs and of regional lymph nodes, leading to more accurate T and N staging.

Finally, similar to standard contrast-enhanced abdominal CT, CTC with intravenous contrast administration allows for the identification of liver metastases (Figure 6), retroperitoneal or iliac lymph node enlargement, and the presence of peritoneal carcinosis (Figure 7). Moreover, scans conducted at the level of the lower pulmonary lobes can identify lung metastases.

CONCLUSION

CTC is a reliable technique to define the precise segmental location of CRC, to establish the presence of synchronous cancers and polyps greater than 10 mm,

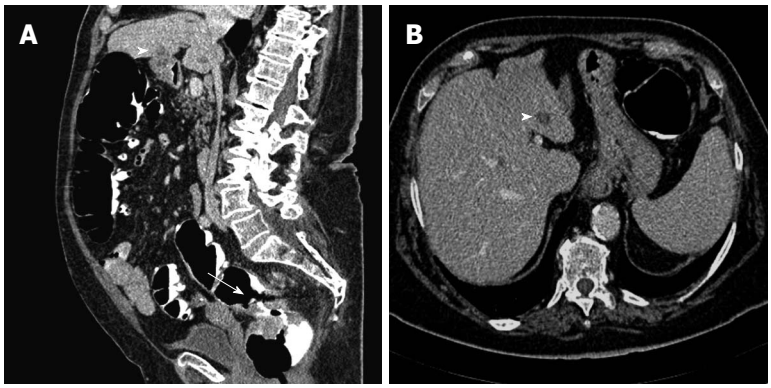

Figure 6 Computed tomography colonography of T3 M1 rectal cancer. A: A sagittal reconstructed image shows the rectal tumor (arrow) and a hepatic metastasis appearing as a hypoattenuating focal lesion (arrowhead); B: An axial source image demonstrates that the metastatic lesion is located in the third hepatic segment (arrowhead).

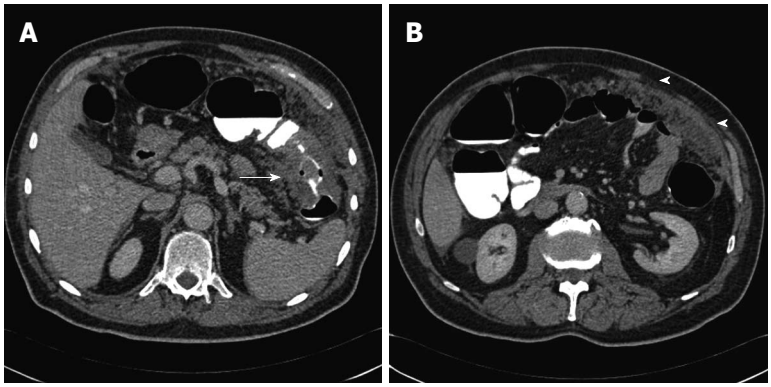

Figure 7 Computed tomography colonography of a T3 M1 stenosing Computed tomography colonography of the transverse colon. A: A Computed tomography colonography (CTC) axial source image shows the lesion (arrow); B: A CTC axial source image depicts marked omental thickening (arrowheads) consistent with peritoneal carcinosis.

and to perform a fairly accurate tumor staging. These factors notwithstanding, CTC has some limitations, including a lack of biopsy capability, suboptimal sensitivity for synchronous small polyps, and unsatisfactory nodal staging. Bearing in mind these limitations, CTC could be employed as a "one-stop-shop" examination for preoperative assessment in patients with CRC.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Proteomics for discovery of candidate colorectal cancer biomarkers

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Author contributions: Álvarez-Chaver P and Martínez-Zorzano VS designed this review; Álvarez-Chaver P, Otero-Estévez O, Páez de la Cadena M, Rodríguez-Berrocal FJ and Martínez-Zorzano VS contributed equally to the writing of this manuscript.
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Abstract

Colorectal cancer (CRC) is the second most common cause of cancer-related deaths in Europe and other Western countries, mainly due to the lack of well-validated clinically useful biomarkers with enough sensitivity and specificity to detect this disease at early stages. Although it is well known that the pathogenesis of CRC is a progressive accumulation of mutations in multiple genes, much less is known at the proteome level. Therefore, in the last years many proteomic studies have been conducted to find new candidate protein biomarkers for diagnosis, prognosis and as therapeutic targets for this malignancy, as well as to elucidate the molecular mechanisms of colorectal carcinogenesis. An important advantage of the proteomic approaches is the capacity to look for multiple differentially expressed proteins in a single study. This review provides an overview of the recent reports describing the different proteomic tools used for the discovery of new protein markers for CRC such as two-dimensional electrophoresis methods, quantitative mass spectrometry-based techniques or protein microarrays. Additionally, we will also focus on the diverse biological samples used for CRC biomarker discovery such as tissue, serum and faeces, besides cell lines and murine models, discussing their advantages and disadvantages, and summarize the most frequently identified candidate CRC markers.

Key words: Colorectal cancer; Serum; Tissue; Proteins; Biomarkers; Proteomics; Protein identification; Mass spectrometry

Core tip: Proteomics is an important tool for the identification of candidate cancer biomarkers since it allows the simultaneous analysis of multiple differentially expressed proteins in a single study. This review provides an overview of recent reports focused on the different proteomic tools used for the discovery of candidate protein markers for colorectal cancer (CRC), such as two-dimensional electrophoresis methods, quantitative mass spectrometry-based techniques or protein microarrays. We also emphasize the use of different samples including cell lines, murine models, clinical samples as tissue, serum or faeces, for CRC biomarker discovery, discussing their advantages and disadvantages, and finally summarize the candidate CRC markers most fre-

quently identified.

Álvarez-Chaver P, Otero-Estévez O, Páez de la Cadena M, Rodríguez-Berrocal FJ, Martínez-Zorzano VS. Proteomics for discovery of candidate colorectal cancer biomarkers. *World J Gastroenterol* 2014; 20(14): 3804-3824 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/3804.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.3804

INTRODUCTION

Colorectal cancer (CRC) is the second most frequently diagnosed cancer and the second most common cause of cancer-related deaths in Europe and other Western countries[1]. This is mainly due to the lack of well-validated and clinically useful biomarkers with adequate sensitivity and specificity to detect this disease at early stages. Over the last two decades CRC survival rates have barely changed, with more than 50% of the patients having regional or distant metastasis at the time of presentation[2].

However, CRC is potentially curable if detected early before the development of metastasis. After the surgical resection of a tumour that is still localized to the colon or rectum (Duke's stage A) patients have a 5-year survival rate of more than 90%. Contrarily, patients with Duke's stage D cancer, where the tumour has spread to other organs, have a 5-year survival rate of less than 10%[2]. Nowadays it is well known that the pathogenesis of CRC is a progressive accumulation of mutations in multiple genes such as *APC*, *KRAS* and *p53*[3]. CRC development is a multi-step process that usually spans about 5-10 years, which offers a period of several years to detect the tumour in an early stage and to interfere with the natural course of the disease[4].

Early detection of CRC can therefore significantly reduce the mortality for this malignancy. However, current screening methods including faecal occult blood test (FOBT), sigmoidoscopy, colonoscopy and virtual computed tomography scanning either lack the required sensitivity and specificity or are costly and invasive[5]. Some biomarkers such as the circulating carcinoembryonic antigen (CEA) levels and tumour-associated gene mutations have only shown some prognostic or predictive value. In particular, the *KRAS* mutation has been proposed as a marker of probable failure of epidermal growth factor receptor (EGFR)-targeted therapy[6]. In patients with metastasis, for whom no curative options remain, therapies include the combination of traditional chemotherapy with the use of new drugs. There is therefore an urgent need for developing new screening tests and identifying new biomarkers to diagnose, predict, and monitor the progress of CRC, and eventually find more efficient drug targets for this disease.

Following the genomics revolution, recent technological advancements allow the proteomic analyses of complex protein mixtures. Proteins, not only genes, are responsible for the phenotypes of cells, therefore it is impossible to elucidate mechanisms of disease solely by studying the genome. Proteomics is the large-scale study of proteins to comprehensively map biological processes such as the molecular mechanisms of carcinogenesis[7]. The proteome is more complex than the genome due to alternative transcription initiation, alternative splicing, RNA editing, proteolytic processing and post-translational modifications (phosphorylation, glycosylation, *etc.*), among others. Therefore, the knowledge of the human proteome is an extraordinary challenge. Proteomics can be defined as the discipline that includes the set of methodologies used for the large-scale study of a proteome, *i.e.* the set of proteins in an organism, a cell or any biological system, in a given time and under certain conditions. It should be noted that proteomics does not focus exclusively on the identification and quantification of these proteins, but also in the study of their location, their modifications, their interactions and their functions.

Proteomic studies generate large protein databases and an expanding list of candidate protein markers that are differentially expressed in CRC patients, identified using two-dimensional electrophoresis (2-DE) and two-dimensional differential in-gel electrophoresis (2D-DIGE) techniques[8]. As an alternative to 2-DE and 2D-DIGE, proteomic studies have also employed the technique of direct expression profiling of tumour and normal tissue by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) or by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)[9]. With these approaches, the reproducible spectra profiles of tumour and normal tissue are used to generate classification models. More recently, the technique of choice is the LC-MS/MS (liquid chromatography-tandem mass spectrometry), as it is more rapid and sensitive. With this methodology, commonly referred to as shotgun analysis, proteins from a complex mixture are collectively in-solution digested and the resulting complex mixture of peptides is separated by high-performance liquid chromatography (HPLC). Then, chromatographic fractions are introduced directly into a sensitive tandem mass spectrometer capable of isolating and fragmenting individual peptides. Protein identification is performed at the level of peptide fragmentation patterns acquired during tandem MS, which are indicative of the amino acid sequence.

Briefly, in this review we provide an overview of recent reports describing the different proteomic techniques used and the diverse biological samples employed for the discovery of new candidate protein markers for CRC.

PROTEOMIC TECHNIQUES FOR COLORECTAL CANCER BIOMARKER DISCOVERY

The analysis of the proteome changes between normal

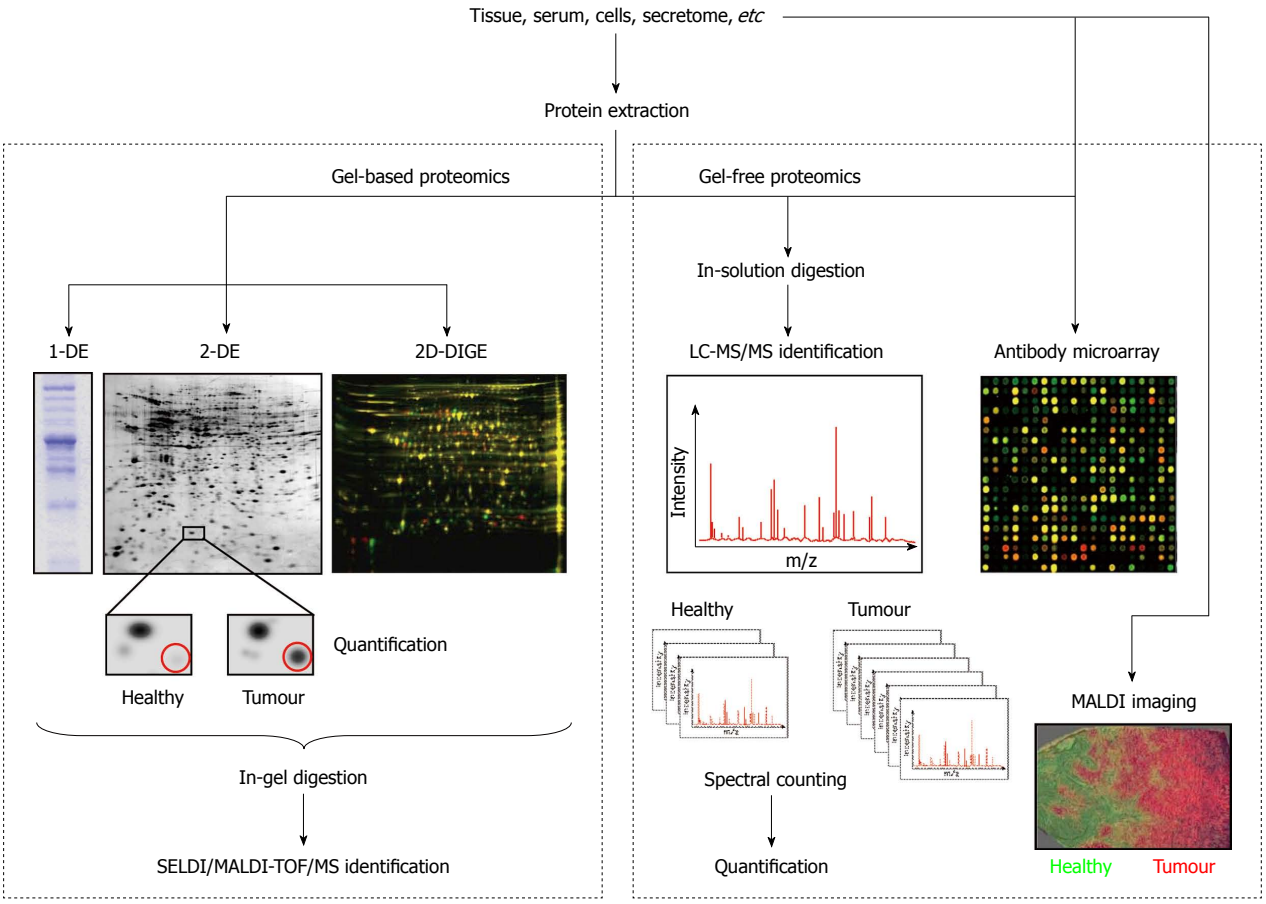

Figure 1 Schematic representation of the principal workflows used in proteomics for colorectal cancer biomarker discovery. Please note that for both gel-based and gel-free proteomics only one of the quantification methods is shown. DE: Dimensional electrophoresis; 2D-DIGE: Two-dimensional differential in-gel electrophoresis; SELDI: Surface-enhanced laser desorption/ionization; MALDI: Matrix-assisted laser desorption/ionization; TOF: Time of flight; MS: Mass spectrometry.

and diseased samples is known as comparative proteomics and is fundamental for the discovery of candidate cancer biomarkers. This area of proteomics employs the techniques described below and the workflows outlined in Figure 1.

1-D electrophoresis

For many proteomic applications, 1-D electrophoresis (1-DE) is the method of choice to resolve protein mixtures. Proteins are solubilised in sodium dodecyl sulphate (SDS) and then separated on the basis of their molecular weight (MW). This technique is simple to perform, is reproducible, and can be used to resolve proteins with molecular masses ranging between 10 and 300 kDa[10]. Due to its limited resolving power, the most common application of 1-DE is the characterization of proteins after a purification procedure[11]. Nowadays, it is also often employed to carry out the digestion of samples to be analysed by LC-MS/MS because it is more effective than in-solution digestion. For example, Lim *et al*[12] employed SDS polyacrylamide gel electrophoresis (PAGE) to overcome the limitation of two-dimensional (2-D) electrophoresis for resolving extreme acidic, basic, or membrane proteins. In their study, the protein bands were subjected to in-gel digestion and protein analysis was performed using electrospray ionization (ESI) ion trap mass spectrometer. Among the differentially expressed proteins they identified low abundant proteins and proteins with extreme pH, which were previously not detected in 2-D gels. In another study, a lectin affinity-based approach followed by the same proteomic strategy (SDS-PAGE coupled to LC-MS/MS) was employed to detect differentially expressed secreted proteins in the secretome (conditioned media) of cultured paired normal and CRC tissues. EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2) was found up-regulated and was further validated by immunohistochemistry (IHC) at tissue level and enzyme-linked immunosorbent assay (ELISA) at serum level. The expression level of EFEMP2 was dramatically increased in CRC patients, even at early stages. Moreover, the diagnostic accuracy of EFEMP2 was superior to that of CEA, with an area under the receiver operating characteristic curve of 0.923 and 0.728, respectively. These authors concluded that EFEMP2 is a promising serum biomarker for the early detection of CRC[13].

2-D electrophoresis

Although 2-DE is commonly described as a laborious technique with low throughput that requires a relatively large amount of sample, its capability to separate thousands of proteins in a single analysis made it a major player in the profiling of protein expression in cancer

and the core technology for protein separation prior MS characterization. In fact, this procedure offers a good resolution because it combines two types of gel electrophoresis techniques: isoelectric point (pI)-based protein separation by isoelectric focusing (IEF) and MW separation by SDS-PAGE.

Traditionally, a comparative proteomic study involves the extraction of the protein content from the samples (tissue, serum, cells, *etc.*) in an appropriate lysis buffer, the separation of samples on 2-D gels, the staining of gels with a protein stain such as Coomassie brilliant blue, silver nitrate or SYPRO, the acquisition of images and the matching of protein spots using a statistical package[14] such as Melanie, PDQuest or Progenesis Samespots. These analyses generate two master 2D maps, one for the healthy samples and another one for the pathological samples. Subsequent analyses with the same software compare the two maps to detect proteins which are present in greater or lesser quantities in one of the samples. Then, differentially expressed proteins are excised and subjected to in-gel digestion with trypsin for MS identification in protein databases.

Using 2-DE, often referred to as gel-based proteomics, protein isoforms and variants expressed by a biological system (tissue, cells, *etc*) may be displayed, allowing the visualization of different phenotypes[15,16]. However, it is clear that classical 2-DE has several limitations. For example, proteins that are low-abundant, or have a MW lower than 10 kDa or higher than 150 kDa, as well as those with very basic pI values, are seldom detected using conventional gels. Moreover, insolubility precludes the penetration inside the gels of hydrophobic or membrane-associated proteins that are of special interest in the biomarker discovery field. However, since its development in the 1970s as the first approach to separate complex protein mixtures, the role of 2-DE in the proteomics workflow has been well preserved due to the emergence of new methodologies and protocols that overcome its limitations. In 1975 the first buffer for protein solubilisation was introduced, but the proposed procedure was not very suitable for membrane-associated, alkaline or hydrophobic proteins separation[14]. To perform a complete protein solubilisation several additives must be included in the buffers, such as chaotropes (urea, thiourea) to prevent protein aggregation, detergents (Triton x 100 or CHAPS) to increase the solubility of certain proteins, and reducing agents such as dithiothreitol (DTT) to reduce disulphide bonds. After disulphide links reduction, the newly produced free sulphidryl groups must be protected by alkylation, being iodoacetamide (IAA) the alkylating reagent most compatible with 2-DE. Moreover, sample solubilisation can be improved by procedures such as agitation and ultra-sonication[17].

Using gel-based proteomics, many studies have been carried out in order to find new CRC biomarkers. Among the most recent we can highlight the work of Chen *et al*[18] who found an overexpression of alpha-enolase, the heat shock protein HSP27 and macrophage migration inhibitory factor (MIF) in tumour tissue of CRC patients with low preoperative serum CEA. They corroborated that serum alpha-enolase and MIF were significantly overexpressed in those patients, improving the diagnosis of primary CRC when combined with the determination of preoperative CEA levels. Other examples of 2D gel-based discovery studies that have yielded novel candidate CRC serum markers include S100A8 and S100A9[19], and desmin[20].

Besides comparisons between normal and tumour-derived tissues, several 2D gel studies have analysed metastatic and non-metastatic CRC tissues and have validated candidates for CRC markers using IHC and functional analyses in cell lines and mouse models. Zhao *et al*[21] performed a comparative proteomic analysis to show that Rho GDP-dissociation inhibitor (RhoGDI) is markedly up-regulated in metastatic CRC, validating the result by Western blot in tissue and cells and by IHC in 126 pathologically characterized CRC cases. RhoGDI was shown to correlate with tumour invasion, lymph node metastasis and clinical stage. Authors also demonstrated that the transfection of the *RhoGDI* gene in HT29 cells with low levels of this inhibitor resulted in an increase in cell proliferation and motility *in vitro*. In another similar study, the same authors showed that gene transfection-mediated overexpression of LIM and SH3 domain protein 1 (LASP-1) in SW480 human colon adenocarcinoma cells resulted in an aggressive phenotype of cancer cells and promoted cancer growth and metastasis[22]. More recently, using 2D serum proteome analysis combined with MS, transthyretin (TTR) was also identified by these authors as a specific marker of CRC metastasis[11]. Other CRC tumour markers, which have been widely studied by our group through the use of 2D technology, are the proteins clusterin[23] and nucleoside diphosphate kinase A (NDK A)[24].

As we have mentioned above, many proteomic studies from the past 10 years have focused on the comparison of colorectal tumour and adjacent normal mucosa tissues. These analyses predominantly employed 2D gel separation of total tissue lysates which limit the loading amount of sample, restricting the analysis to the more abundant proteins that mask minor proteins that could be interesting as possible CRC marker candidates. In an attempt to identify less abundant CRC proteins, few studies have combined more targeted approaches with 2-DE, including studies focusing on membrane proteins[25,26] or basic proteins[27,28]. Despite these targeted attempts, the analyses still are largely limited to abundant proteins that are found overexpressed in several tumours, such as structural proteins, glycolytic enzymes or heat shock proteins. In serum 2D analysis, the previous depletion of albumin and IgG, which account for more than 60% of the total serum proteins, may result in the loss of potentially important proteins bounded to them. Therefore, sample preparation improvements like sonication before the depletion and desalting steps allowed the detection of valuable, low abundant proteins in serum of CRC

patients[11]. Other improvements of 2-DE were the introduction of new gels which can separate proteins with extreme pI values and/or make use of narrow range pH gradients for increased resolution, as well as the use of improved pre-fractionation strategies. Consequently, 2-DE remains as a pivotal methodology for the display of an extensive image of a complex mixture of proteins.

Differential in-gel electrophoresis

An exciting advance in 2-DE, which improved the speed and reproducibility of conventional 2-DE, was introduced by Unlü *et al*[29]. This technology is called differential in-gel electrophoresis (DIGE). Basically, different protein samples (healthy *vs* pathological, for example) are labelled on lysine side chains with succinimidyl esters of propyl-Cy3 and methyl-Cy5, two fluorophores that emit light at different wavelengths (Figure 1). An internal standard is prepared by pooling equal amounts of samples, labelled with a third dye (Cy2). The protein samples are mixed prior to separation and loaded onto the 2D gel together. After electrophoresis, the 2-D pattern is visualized by imaging the gel with a fluorescence scanner by sequential excitation of the fluorescent dyes. Three images are obtained, which are combined to identify pattern differences. Because the samples run together, differences in gel preparation, running conditions and local gel structure are eliminated, making this technique of great utility for biomarker studies. However, 2D-DIGE also has its drawbacks: fluorescent labels are less sensitive than both SYPRO dyes and silver staining, proteins differ in their labelling efficiency, and the technique is relatively expensive compared to silver or Coomassie staining of gels.

Using 2D-DIGE, proteome analysis of membrane fractions in colorectal carcinomas revealed several proteins with an altered expression[26]. Among them, annexins (A2, A4, A5 and A7), lamin B, calponin 1 and voltage-dependent anion channel (VDAC) were analysed by IHC using tissue microarrays. Authors proposed annexin A2, annexin A4 and VDAC as candidate markers for colorectal cancer diagnosis and, presumably, also for therapy. More recently, Ma *et al*[30] used 2D-DIGE coupled with MS to screen for biomarker candidates in the serum proteome of CRC patients and healthy donors. They identified and validated transaldolase 1 and thyroid receptor interactor as CRC-associated serum biomarkers. Sawhney *et al*[31] demonstrated the compatibility between the subcellular fractionation by laser microdissection (LMD) of human colon tissue and 2D-DIGE. They observed a greater coverage of proteins from very small amounts of micro-dissected material. Sugihara *et al*[32] compared the proteome of normal colorectal epithelial tissues with that of the tumour in 59 CRC patients using 2D-DIGE. They found a higher expression of 110 protein spots and focused on the adenoma polyposis coli-binding protein (EB1). This protein was originally discovered as a binding protein of the tumour suppressor gene product APC, and had been associated with poor prognosis in several malignancies but not in CRC. Immunohistochemical analysis of 132 CRC cases revealed that EB1 was overexpressed in tumour cells and was correlated with poor prognosis. Therefore, they proposed EB1 as a candidate biomarker and therapeutic target for CRC. In another study, the proteomic analysis of six paired normal and CRC tissues by 2D-DIGE and MALDI-TOF-MS showed two markedly down-regulated proteins, which were identified as cytoplasmic carbonic anhydrase I and Ⅱ and whose changes were further validated by IHC and Western blot. The down-regulation of these enzymes is an early event in colorectal carcinogenesis, but is not correlated with lymph node metastasis[33]. More recently, Zhou *et al*[34] showed that overexpression of carbonic anhydrase Ⅱ remarkably suppressed tumour cell growth both *in vitro* and *in vivo*. Using the Caco-2 cell line, an *in vitro* model to study colorectal carcinogenesis, our research group identified the translationally-controlled tumour protein (TCTP) and the transforming growth factor-β-induced protein ig-h3 (TGFβ1p), among others, as candidate biomarkers for CRC[35]. Grandjean *et al*[36] used the new methodology of sequential immunoaffinity depletion-differential in gel electrophoresis (SID-DIGE), that allowed the efficient screening of sera for the identification of autoantibodies as candidate biomarkers. The identification of autoantibodies is based on the characterization of tumour-associated antigens against which they are directed. Among the 25 tumour-associated antigens identified, 7 were also detected using the conventional SERPA (serological proteome analysis) technique, validating their new approach. The identification of the additional 18 autoantibodies proved the potential of this new method.

Protein microarrays

Using protein microarrays, the simultaneous analysis of different proteins is performed in one single experiment, allowing the study of the protein identity, quantity, interaction and function. There are two types of protein arrays: forward-phase protein arrays and reverse-phase protein arrays.

For forward-phase protein microarrays (also known as capture arrays), the elements of the array are capture molecules (antibodies, proteins, nucleotides or aptamers), each binding specifically to a particular protein. Antibody arrays are the most common, and use antibodies immobilized on a solid surface or membrane to specifically interact with the proteins of interest. For the detection, samples can be labelled with different fluorophores like in 2D-DIGE (Figure 1), allowing two possible samples to undergo the same treatment for comparison[37]. This technology has started to be implemented extensively in cancer research. For example, Ellmark *et al*[38] prepared a cell suspension from a colorectal tumour containing a mixed population of cells which was captured on an antibody microarray. Cancer cells were detected using a fluorescently tagged antibody for carcinoembryonic antigen (CEA-Alexa647) or epithelial cell adhesion molecule (EpCAM-Alexa488). Using this multiplexing procedure, authors found a differential expression of CD45, CD71

and CEA in cancer cells, among others proteins.

In a reverse-phase protein microarray, the samples are immobilized on the surface or membrane and antibodies are then be applied to the array to detect specific epitopes, protein sequences or structures[37]. For example, Oliveira *et al*[39] used tissue microarrays and found that NDK A protein expression was higher in tumour tissue of CRC patients than in adjacent non-neoplastic mucosa. In another study, serum CRC biomarker candidates Apolipoprotein AI (Apo A1) and C9 complement component (C9) were selected by liquid chromatography (LC) and MS, and then validated using a reverse-phase protein microarray[40].

Currently, antibody microarrays are attracting considerable attention in cancer biomarker discovery. Several aspects of microarray technology make it well suited to cancer research because of the low-volume requirements and its multiplexed detection capability that make optimal use of precious clinical samples. These assays are rapid and highly amenable to automation, which makes them ideal for biomarker studies[41]. For a review of array-based detection of serum autoantibodies in CRC the reader is referred to Tan *et al*[42]. In addition, the equipment of SPR (Surface Plasmon Resonance) is now available for the analysis of protein microarrays, allowing the study of interactions and the identification by MS of ligands of interest. A protein array variant vastly used in the search for new biomarkers for CCR is the technique named Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (SELDI-TOF-MS). It is a widely used technology platform for biomarker discovery in tissue, plasma and serum, though it is most commonly associated with the development of serum-based markers. SELDI-TOF-MS combines two powerful technologies, chromatography and MS, and consists of solid supports or chips made of aluminium or stainless steel coated with specific chromatographic surfaces, including reverse phase, anionic exchange, cationic exchange and immobilised metal affinity surface. In the case of serum samples, the prefractionation technique called ProteoMiner™ (Bio-Rad Laboratories, Hercules, CA, United States) is widely used prior to the analysis by SELDI-TOF-MS due to the presence of a wide range of protein concentrations.

Mass spectrometry-based proteomics

For the protein identification by MS two strategies can be pursued. On one hand the so-called peptide mass fingerprinting (PMF) and on the other hand, the sequencing or tandem mass spectrometry (MS/MS). In both cases it is necessary to digest the protein, and its fragments (peptides) should pass to a gas state. At the end of the 1980s, two revolutionary methods for the ionization of peptides and proteins were developed: the electrospray ionization (ESI) and the matrix-assisted laser desorption/ionization (MALDI), for which the authors received the Nobel Prize in Chemistry in 2002[43].

Less than five years ago, the strategy used in most of the proteomic studies aimed at the search of new tumour markers was firstly the separation of the proteins in the sample using 2-DE or 2D-DIGE to compare the proteome between healthy and tumour tissues and, secondly, the removal of the spots of interest from the gel for the subsequent identification by SELDI/MALDI-TOF-MS[44]. However, as an alternative to 2-DE and 2D-DIGE, most of the proteomic studies in the last three years have employed another technique of MS to carry out studies of differential expression between samples from healthy individuals and patients: the liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS). This methodology allows performing a previous separation of the peptides typically in a reverse phase chromatographic column. As the LC equipment is usually connected on-line to the mass spectrometer, the fractions obtained after the chromatography enter successively in the mass analyser, allowing the one by one slicing of the peptides present in the sample. This analysis can be performed with digested bands from a 1-D gel, with digested spots from a 2-D gel or with a complex mixture of proteins not previously separated so that the peptides from different proteins are mixed after digestion. This latter type of analysis is known as shot-gun proteomics or multidimensional protein identification technology (MudPIT). However, prior to the MS analysis, it is necessary to perform two independent chromatographic separations in different conditions to resolve the complexity of the sample. In addition, mass spectrometers used for this type of studies have great resolution and sensitivity, obtaining a much higher number of proteins identified compared to that obtained with classical proteomic studies based on 2-DE or 2D-DIGE[45]. Several authors have described the advantages of this technology in comparison with gel-based proteomics. For example, the proteasome activator complex subunit 3 (PSME3), an intracellular CRC-associated protein, was identified in a 2D gel-based study comparing normal and cancer tissues. This candidate was then selected for follow-up analysis by immunoblotting[46]. One year later, in a second study of the same authors, PSME3 was validated as a novel CRC serum marker using MS[47]. This new study detailed the technical advantages of the mass spectrometry-based approach for relative quantification of protein abundance, as compared to the traditional image analysis approach. Importantly, PSME3 up-regulation would have been missed by image analysis because this protein was masked by another co-migrating high-abundant protein, annexin A4. As another interesting example, Thierolf *et al*[48] analysed the same paired normal and CRC tissues by 2-DE and 2D-LC-MS/MS, showing the complementarity of these two different strategies since a set of proteins were uniquely identified by each one. Among the identified proteins S100A12 was validated in serum, and authors concluded that this protein was more sensitive for the detection of CRC patients than CEA.

Another MS approach, the MALDI-TOF-MS, is most commonly used to discriminate tumour from normal

tissue and in some cases can sub-classify disease. For example, Liao *et al*[49] evaluated the potential value of this approach to classify various clinic-pathological features in CRC. They found 73 protein peaks with a higher expression in tumours than in adjacent normal mucosa tissues in the mass range of 1800-16000 Da. Using "leave-one-out" cross validation algorithms for tumour spectra they correctly classified poorly, well and moderately differentiated tumours. Similar analyses of normal mucosa spectra correctly predicted disease recurrence, disease-free survival and metastasis. More recently, Fan *et al*[50] employed a well-defined technology platform called ClinProt (Bruker Daltonics, Germany), based on magnetic bead purification of peptides and MALDI-TOF-MS. They successfully detected 61 short peptides, from 1 to 18 kDa, which were differentially expressed in the serum of patients with CRC, concluding that this peptidome pattern may provide an alternative for CRC diagnosis or may help in tailoring the use of chemotherapy to each patient. MALDI is also a method of ionization used for analysing the molecular content of tissue sections, including formalin-fixed and paraffin-embedded tissue samples, which are the standard embedding techniques used in clinical routines. This technique is named imaging mass spectrometry (MALDI-IMS), commonly known as MALDI imaging. It has emerged in the last few years as a useful tool for the molecular classification of tissue samples regarding disease stage, risk stratification and therapy response, as well as for the identification of disease biomarkers[51]. Recently, this approach was employed to evaluate fresh frozen sections of CRC tissue and adjacent healthy mucosa, offering novel insights into tumour micro-environmental biochemistry[52].

SELDI-TOF-MS

As mentioned above, SELDI-TOF-MS is a widely used technology platform for biomarker discovery. It can be described as a type of MALDI-TOF-MS where the sample matrix, known as protein chip, has an active role in the sample purification as well as in the desorption/ionization step[53].

Several peptide-profiling studies using serum of CRC patients have reported the combination of SELDI-TOF-MS with bioinformatics in order to perform pattern diagnostics. For an overview of these studies, readers can refer to the work of Gemoll *et al*[54]. As an example, Liu *et al*[55] determined a set of two protein peaks that had the ability to distinguish patients with different stages of CRC from healthy subjects, with a sensitivity of 95.0% and a specificity of 94.9%. More recently, Helgason *et al*[56] identified 13 candidate biomarkers for CRC (m/z 2.0-31.9 kDa) and 2 peaks (m/z 2022 and 28100 Da) that changed during chemotherapy in accordance with patient response to the treatment. Nevertheless, when human serum or plasma is studied with this technique, its sensitivity is restricted due to the wide dynamic range of serum protein concentrations. In this context, sample pre-fractionation targeting the low abundant proteins may help to overcome these limitations. Therefore, the combination of ProteoMiner ™ (Bio-Rad Laboratories, Hercules, CA, United States) pre-fractionation and SELDI based protein profiling is suitable for large-scale serum proteome profiling studies yielding reliable and reproducible results[57].

In tissue studies, only Melle *et al*[58] have reported the detection of differences between normal mucosa and colorectal carcinoma by SELDI. In 2005 they found that pituitary adenylate cyclase activating polypeptide precursor (PACAP), heterogeneous nuclear ribonucleoproteína A1 (HNRNP A1), flavin reductase, calgizzarin (S100A11), nucleoside diphosphate kinase B (NDK B), cyclophilin A and smooth muscle protein 22-alpha showed significantly differential abundance between colorectal tumour tissue and adjacent normal mucosa. In another study published a year later, these authors validated the differential expression of the calgizzarin (S100A11) by immunological techniques[59]. Regarding other clinical material, Ward *et al*[60] used urine of CRC patients as sample to detect proteomic changes in MALDI and SELDI spectra. They found a number of changes in peak intensity significantly associated with colon cancer and these, in conjunction with class prediction models, yielded a diagnostic sensitivity of 78% and a specificity of 87% (values higher than those obtained with serum CEA).

MS-based strategies for protein quantification

Although the quantification in gel-based approaches as 2-DE and 2D-DIGE is very accurate and sensitive, the relative high amount of protein sample necessary for protein identification, as well as the multiple experimental steps required, are the major disadvantages of these techniques. Due to these drawbacks and as a consequence of the technical improvements in the fields of chromatography and mass spectrometry, novel MS-based quantification strategies have been developed, allowing high throughput proteome analyses complementary to gel-based approaches, leading to a higher proteome coverage.

Current MS-based strategies for protein quantification can be divided into two main groups: strategies based on labelling a specific amino acid residue and the so-called label-free proteomics. In all labelling strategies the first and maybe the most critical step is to modify the molecular mass of a specific amino acid so it can be distinguished from its unlabelled counterpart in the detection phase. This can be done in various ways: in one approach stable isotope labelling is used without changing the chemical identity of the amino acid, as in the case of introducing stable isotopes of ^{2}H, ^{13}C, ^{15}N and ^{18}O within various functional groups. In a second approach, chemical modification with or without stable isotope labelling is used. Alkylating Cys is an example of the first case, while guanidination which transfers C-terminal Lys to homoarginine is an example of the latter[61]. Basically, proteins or peptides in one of the samples are modified with an isotope tag. The two samples are then mixed before being processed and analysed by MS. Although the

physical characteristics of the peptides remain the same, the masses of the isotopically labelled peptides are proportionally shifted in the spectrum, and the ratio between the intensities of the differentially labelled peptides peaks then permits accurate relative quantitation of the proteins. Stable isotopes can be incorporated into proteins or peptides using different techniques: O^{18} proteolytic labelling, isotope-coded affinity tags (ICAT), isotope-coded protein labelling (ICPL), iTRAQ (isobaric Tags for Relative and Absolute Quantification), or SILAC (Stable Isotope Labelling with Amino acids in cell Culture), where stable isotopes are incorporated into growing cells. The advantage of labelling strategies is that in the same experiment several samples can be analysed and very small changes of expression can be detected. For example, Kim *et al*[62] combined 2-DE with ICAT and found a set of five proteins (VCP, TPM2, ITLN1, TAGLN and FABP1) differentially expressed in the tumour tissue of CRC patients, with value for the prognosis of the disease. Using the iTRAQ-based quantitative proteomics approach Ghosh *et al*[63] validated the role of calcyclin binding protein (CacyBP) in promoting colorectal cancer metastasis.

The goal in the search for new CRC biomarkers is not the identification of proteins with small changes of expression; instead, large variations in their expression are desirable. Therefore, proteomic techniques based on LC-MS/MS without prior marking of the samples (label-free proteomics) are frequently used, despite these are time-consuming since the samples are analysed one by one. Using this approach with the insoluble fractions from tissues from CRC patients, Yang *et al*[64] found four proteins (KRT5, JUP, TUBB, and COL6A1) that gave specific network information for CRC. Their panel of novel markers proposed as candidate targets for treatment was further validated by Western-blotting. By label-free quantitative mass spectrometry and protein microarray, Matsubara *et al*[65] reported that adipophilin is a plasma biomarker potentially useful for the detection of early-stage CRC, showing improved diagnostic performance.

A novel label-free quantitative proteomics technique is the so-called SRM (Selected Reaction Monitoring). It is an MS/MS method, which consists in selecting a specific precursor ion or peptide that will later be divided, allowing the selection of one of its product ions. This detection is used to make a relative or absolute quantification of the peptide and, by extension, of the protein to which it belongs in the analysed sample. Although this approach can be used without making a prior labelling, a recent study combined this technique with ICAT to compare healthy mucosa and tumour tissues of CRC patients, identifying more than 1000 proteins overexpressed in tumours, related to endocytosis, mitochondrial dysfunction and various cell signalling pathways[66]. Other MS-based strategy for protein quantification is label-free spectral counting. Considering that the detected spectral counts are correlated with the abundance of corresponding proteins, Yao *et al*[13] compared and identified proteins in the secretome of paired normal and CRC cultured tissues before and after a lectin capture method. After lectin capture the percentage of spectral counts of secreted proteins was significantly increased from 45% to 80% for the conditioned medium of normal tissues, and from 50% to 85% for the CRC ones, indicating that secreted proteins were effectively enriched by the lectin affinity based approach.

BIOLOGICAL SAMPLES FOR COLORECTAL CANCER BIOMARKER DISCOVERY

The first critical issue in the proteomic analysis for CRC biomarker discovery is the selection of the sample. Different samples can be used for searching candidate protein markers, including clinical samples such as serum, tissue or faeces from patients, as well as other biological samples like cell lines or animal models. Below, we will briefly discuss the advantages and disadvantages of these different types of samples, and summarize the putative CRC markers most frequently identified.

Serum or plasma

Blood is the most suitable sample used to identify biomarkers for the screening or diagnosis of CRC due to its availability and non-invasive collection. However, biomarker detection in serum or plasma has some drawbacks. One is the fact that in these fluids a heterogeneous mixture of proteins derived from different tissues is found, making it difficult to attribute a differentially expressed protein to a tissue-specific disease. This limitation can result in the identification of putative protein markers that are not specific for CRC. Furthermore, in serum a small number of major proteins (*e.g.*, albumin) are highly concentrated and mask other less abundant proteins that could be interesting as biomarkers. One strategy to overcome this problem is to remove the serum most abundant proteins by using commercial affinity columns designed specifically for this purpose[67].

Despite these shortcomings, in the last years several proteomic studies have been published focusing on the search of serum proteins to discriminate CRC patients from healthy individuals. Interestingly, different authors including our own group identified the same differentially expressed proteins using a variety of proteomic tools[18,19,40,68-74]. Among those proteins we found apolipoproteins, cathepsins, alpha-1 antitrypsin, alpha-1-acid glycoprotein types 1 and 2, transferrin, beta-2 microglobulin, complement components C3 and C9, gelsolin, heat shock proteins as HSP60, transthyretin, defensins (also known as human neutrophil peptides), alpha-enolase, S100 A calcium binding proteins, as well as the inhibitor of metalloproteases TIMP-1. This latter marker is known to be aberrantly glycosylated in patients with CRC[75].

Recently, the application of new proteomic technolo-

gies allowed the identification of other candidate proteins such as adipophilin, also known as perilipin-2 or adipose differentiation-related protein[65], and kininogen-1[76], for the early detection of CRC. Although in some cases these putative markers show good diagnostic parameters, in general it has been demonstrated that a single protein marker is not enough to get high sensitivity and specificity values for CRC diagnosis. Therefore, in order to reach a greater diagnostic accuracy it can be more useful to use a panel of several markers. In fact, Brock *et al*[77] have described that the protein panel alpha-1-acid glycoprotein 1, gelsolin, C3, C9, hyaluronic acid binding protein 2 (HABP2) and serum amiloide A2 (SAA2) yielded a sensitivity of 94% and a specificity of 83% to differentiate CRC patients from healthy donors.

Looking for the identification of metastasis-associated proteins, the serum of healthy individuals *vs* patients with metastatic CRC were compared. Specific proteins were identified in the patients, including mitogen activated protein kinase activated protein kinase 3 (MAPKAPK3), activin A receptor ⅡB (ACVR2B), Pim-1 oncogene (PIM-1), v-src sarcoma viral oncogene homolog (SRC) and fibroblast growth factor receptor-4 (FGFR4)[78]. Other authors have compared serum samples from CRC patients with lymph node or liver metastasis and those from patients without recurrence or metastasis for at least 3 years. In these studies the protein transthyretin (TTR) was identified as a candidate lymph node metastasis marker in CRC[11], whereas a panel of 8 peptides identified as fragments of alpha-fetoprotein, complement C4-A, fibrinogen alpha, eukaryotic peptide chain release factor GTP-binding subunit ERF3B, and angiotensinogen, demonstrated promising value for predicting liver metastasis in patients who underwent radical resection of CRC[79].

Tissue

The analysis of tissue samples obtained from CRC patients allows comparing the protein profile between tumour and the adjacent healthy mucosa, particularly useful in the discovery of prognostic tumour markers. This approach has several advantages over serum or plasma analysis. The first one is that not all the proteins altered in the tumour, and therefore marker candidates, are secreted to the blood from the tumour cells. Therefore, the concentration of a putative marker is higher in tumour tissue than in blood. In addition, since tumour samples are analysed, there is no doubt that the altered proteins identified originate in the tumour itself. However, proteomic studies in tumours have also some disadvantages. First of all, the availability of this type of sample is limited. Moreover, tumours are heterogeneously composed of neoplastic cells besides the surrounding stromal cells. As an example of this latter drawback, various studies have described alterations of the protein vimentin in colorectal tumours[80-82] even though this protein is not expressed in epithelial cells, only in stromal cells and lymphocytes. Therefore, the identification of specific biomarkers would require the isolation of the tumour epithelial cells. However, because of the small size of tissue biopsies and other technical reasons, this separation is usually not carried out. In consequence, some proteins overexpressed in tumours are also increased in inflammation and this lack of specificity may prevent its use in clinical practice.

An overview of the proteins with altered expression in colorectal tumours identified by different authors comparing matched normal and tumour tissues reveals that many of them are abundant proteins such as structural and cytoskeleton proteins, annexins, chaperones or glycolytic enzymes which are also modified in other cancers[83]. In order to detect less abundant proteins, an approach that will be discussed later in this review is the isolation of subcellular fractions and the characterization of the corresponding sub-proteomes.

Among the proteins differentially expressed in colorectal tumours and consistently described in literature as candidate markers we can highlight translationally controlled tumour protein (TCTP)[21,46,84], NDK A[19,20,46,84] and S100A9[19,85], all of them overexpressed in tumours, as well as selenium-binding protein (SELENBP1 protein)[19,85] and carbonic anhydrase 1 (CA-1)[27,85,86] which are less expressed in the tumour tissue. Noticeably, in some cases the analysis of whole tumour lysates has led to the identification of marker candidates which have been subsequently validated in tissue or serum samples from a different cohort of CRC patients. Among these interesting biomarkers are proteins from the S100A family such as S100A8, S100A9[19] and S100A12[48], ribonucleoprotein HNRNPA1[87], the enzyme nicotinamide *N*-methyl transferase (NNMT)[46], proteasome activator complex subunit 3 (PSME3)[47], desmin[20] and olfactomedin-4 (OLF4). This latter protein is overexpressed not only in adenocarcinomas at early stages, but also in adenomas[88].

Besides the comparisons between healthy mucosa and tumour tissue, other authors have analysed metastatic and non-metastatic CRC tissues. Kang *et al*[89] compared the primary tumour tissues from 14 CRC patients with and without hepatic metastasis. They found a differential protein cluster, consisting of 17 proteins from the PI3K/AKT pathway which was validated by Western blot. A similar approach was performed by Zhao *et al*[21] who detected that Rho GDP-dissociation inhibitor alpha is markedly up-regulated in metastatic CRC.

A different approach to analyse whole tumour proteins has been recently published by Wiśniewski *et al*[90]. In this study, using formalin-fixed paraffin-embedded tissues, they were able to identify about 10000 proteins, many of them previously described as candidate CRC markers. This method offers a great advantage for the identification of prognostic markers since it allows retrospective studies analysing archival paraffin-embedded samples from patients who have been clinically followed-up for several years.

Stool

Stool samples provide important advantages over other clinical samples in the search for CRC markers such as

the availability and the non-invasive collection of the sample. Furthermore, tumour-derived proteins may be more abundant in faeces than in blood. However, one drawback of the proteomic analysis of stool samples is the proteolysis caused by the gut micro-biota, leading to protein degradation.

In faeces of CRC patients the following proteins have been identified: defensins, S100A calcium binding proteins (including calprotectin), haemoglobin, haptoglobin, alpha-1 antitrypsin, lactoferrin, CEA, dipeptidyl peptidases I and Ⅳ, cadherin 17, SELENBP1, pyruvate kinase type M2 (M2PK), metalloprotease 9 (MMP9) and its inhibitor TIMP1[91]. Although some of these proteins are related with inflammation, resulting inespecific for CRC, others have been previously reported as CRC-associated proteins and have been tested as candidate CRC screening biomarkers. Interestingly, tumour pyruvate kinase type M2 showed good sensitivity for the detection of tumours (85%) but not for adenomas (28%)[92].

Moreover, it has been demonstrated that a marker panel containing the proteins S100A12, TIMP1 and haemoglobin-haptoglobin showed higher sensitivity than faecal occult blood, and identified 74% of the patients at early stages of tumour development[93]. More recently, Ang *et al*[94] developed a multiplex analysis for 40 human proteins on faecal samples from eight CRC patient and seven healthy volunteers. These authors identified 24 proteins consistently found in all samples and nine proteins (alpha-1 antitrypsin, alpha 1-acid glycoprotein, complement C3, fibrinogen, haptoglobin, hemoglobin alpha, hemoglobin beta, myeloblastin and transferrin) detected only in the CRC patients. The relatively high abundance of these nine candidate markers in the CRC patient samples indicate that they could be clearly differentiated from the healthy controls.

Cell lines

Cell lines derived from colon tumours offer several advantages over clinical samples for biomarker identification. Cell lines are homogeneous cell populations, they are easy to work with and their availability is almost unlimited. Besides, it is easier to obtain subcellular fractions such as plasma membrane, nucleus, secretome (the conditioned media in which the cells grow), and exosomes from cell cultures. Thereby, cellular proteome complexity is reduced, simplifying the identification of less abundant proteins. Nevertheless, the use of cell cultures has some limitations. Almost all available human colon cell lines come from tumours, whereas cell lines derived from normal mucosa epithelial cells do not exist. This fact prevents the study of the proteome changes associated to the CRC malignant transformation sequence: normal mucosa-adenoma-adenocarcinoma. In addition, cell cultures do not exactly represent the *in vivo* situation since they lack features of an *in situ* tumour such as the interactions among tumoral cells, stromal cells and the immune system. For this reason, it is mandatory that the candidate biomarkers discovered in cell lines are eventually validated in clinical samples from CRC patients[67].

The human colon adenocarcinoma Caco-2 cell line is an accepted *in vitro* model to study colorectal tumorigenesis since Caco-2 cells seems to lose their carcinogenic phenotype during the differentiation process. Taking into account this advantage, several authors have applied different proteomic technologies to compare the whole cell lysates before and after differentiation of Caco-2 cells. Along the differentiation process, the proteins glutathione *S*-transferase alpha 1 (GSTA1), annexin A4 (ANXA4), villin 1 (VIL1), galectin (LGALS3) and phosphoglycerate kinase 1 (PGK1) were up-regulated, whereas the proteins CDC2, PCNA and HNRNPH3 were down-regulated[95,96]. Fanayan *et al*[97] analysed cell lysates of other human colon cancer cell lines (LIM1215, LIM1899 and LIM2405) that were selected to represent a wide range of pathological states of colorectal cancer. They identified both cancer-associated proteins with differential expression patterns, as well as protein networks and pathways which appear to be de-regulated in these cell lines. Examples of candidate markers include mortalin, nucleophosmin, ezrin, alpha and beta forms of spectrin, exportin, the carcinoembryonic antigen family, EGFR and met-proto-oncogene (MET).

In order to identify metastasis-associated proteins, some studies have compared two cell lines with different metastatic potential as SW-480, derived from a Dukes' stage B colon carcinoma and SW-620, derived from a lymph node metastasis of the same patient. Ghosh *et al*[98] analysed the whole cell proteome profiles of these two isogenic colorectal cancer cell lines and identified 147 proteins significantly altered in the metastatic cell. Up-regulated proteins in the SW620 cell line included stathmin, villin, myosin 10 and myristoylated alanine-rich C-kinase substrate (MARCKS), whereas down-regulated proteins included those related to cytoskeletal signalling (type 1 cytoskeletal 13, type Ⅱ cytoskeletal 5, tubulin beta 2A/2B, actin, and several acting binding proteins), annexin A1 and proteins with roles in cellular adhesion including beta-catenin and neural cell adhesion molecule1 (NCAM1). However, a beta-catenin degrading protein, calcyclin-binding protein (CacyBP), was found up-regulated in SW620 cells, suggesting the possible involvement of CacyBP in CRC metastasis through the alteration of beta-catenin-mediated cellular adhesion.

In recent years, the proteomic analysis of the secretome of human colon adenocarcinoma cell lines has been described as a useful strategy for the detection of candidate blood-based markers. This is due because the analysis of a cell-specific secretome limits the contamination by the major proteins of the human serum, favouring the identification of tissue-specific proteins[99]. However, as Malard *et al*[100] pointed out, the analysis of human cell line secretomes by proteomics techniques presents some drawbacks. First, proteins secreted in culture media are highly diluted. Moreover, changes in the physiology of the cells may occur upon a change of medium, as most of secretome studies are performed with the basal me-

dium without the addition of fetal calf serum.

Secretomes comprise protein released through different mechanisms, including secreted membrane vesicles (exosomes) able to transfer information to target cells. Searching for CRC-specific markers, Wu *et al*[101] studied the secretome of 21 cancer cell lines from 12 different cancer types, and found that collapsin response mediator protein-2 (CRMP-2) was exclusively detected in the colon cell lines Colo205 and SW480. This protein was eventually validated in serum, demonstrating its value for discriminating CRC patients from healthy donors. Aiming to find secreted metastasis-associated proteins, other studies have compared the secretome of SW-480 and SW-620 cells. Among the proteins differentially expressed between these two cell lines the proteins alpha 5 and alpha 6 integrins, peroxiredoxins 2 and 6, soluble E-cadherin, growth/differentiation factor GDF15 and trefoil factor 3 (TFF3) have been found[71,102]. Noticeably, the serum levels of GDF15 and TFF3 were significantly increased in CRC patients with lymph node metastasis as compared to patients without metastasis or healthy donors[71]. Besides, another study showed that serum levels of soluble E-cadherin reflect the disease status of CRC patients, although validation in a larger cohort would be required to confirm this finding[103].

Subcellular fractions

As we have previously mentioned, the analysis of whole cell lysates can yield good candidate markers, although the complexity of such a sample may hamper biomarker discovery. An alternative for reducing sample complexity of tissue biopsies or cell lysates is to perform a previous subcellular fractionation of the sample and then to analyse the sub-proteome of different subcellular fractions. In this regard, Yang *et al*[64] focused on the identification of proteins in the insoluble fraction of biopsies from 13 CRC patients and found a panel of four proteins (KRT5, JUP, TUBB and COL6A1) as candidate CRC biomarkers. Other authors have analysed the nuclear matrix proteome comparing adenomas and adenocarcinomas since nuclear phenotypic alterations and chromosomal instability are a hallmark of tumour cells[104]. Therefore, it is expected that among the differentially expressed proteins identified in that study (MNDA, HNRPH2, DNASE1, NUP62, NUP88), some meaningful tumour markers could be eventually validated in larger cohorts.

A subcellular fraction of particular interest that has been studied by several researchers including our own group is the membrane fraction. Membrane-associated proteins account for approximately 30% of human proteome and play essential roles in cell-cell and cell-extracellular matrix interactions as well as in signal transduction. Most of the biomarkers currently used in clinical practice (*e.g.*, CEA) and about 70% of all known therapeutic targets are membrane proteins. However, because of the difficulties to isolate and analyse these proteins, mainly due to their hydrophobic character, there are only few proteomic studies analysing membrane-associated proteins in CRC patients. Comparing paired tumours and healthy mucosa samples the overexpression of several membrane proteins including CEA, claudin-3 and the A1 antigen of the histocompatibility complex has been reported in tumours[105]. Another membrane protein identified as a possible biomarker is STOML2 (stomatin-like protein 2), which was found increased in tumours and also detected in serum of CRC patients at early stages of the disease[106]. Other authors have identified candidate markers for metastasis when comparing the cell surface proteomes of the low metastatic cell line KM12C and the high metastatic KM12SM cells. A number of cell signalling, integrins and other cell adhesion proteins (cadherin 17, junction plakoglobin) were described among the most de-regulated proteins[107].

Our research group has analysed the sub-proteomes of membrane and soluble fractions of tumours, and compared them with those from adjacent healthy mucosa. Among the de-regulated membrane proteins we found cytoskeleton proteins, chaperones and two isoforms of the calcium binding protein S100A6[81]. Regarding the soluble fraction a panel of four putative biomarker proteins were identified, 14-3-3 protein zeta/delta, parkinson protein 7 (DJ-1), retinoblastoma binding protein 4 (RBBP-4) and nucleoside diphosphate kinase A (NDK A), differentially expressed in normal and tumour tissues. Furthermore, NDK A was detected in serum and preliminary results indicated an increased concentration in CRC patients. Thus NDK A is an interesting candidate serum biomarker for CRC[24].

Exosomes are 40-100-nm diameter membrane vesicles of endocytic origin that are released from most cell types and circulate in body fluids, including blood. Therefore, analysing the protein profile of these vesicles released by tumour cells can be a useful method to identify markers for the diagnosis of CRC. Indeed, the proteome analysis of exosomes derived from tumoral colon cell lines has led to the identification of the candidate markers claudin-3, Ephrin-B1 and galectin 4[108], cadherin-17, CEA, EGFR, glycoprotein A33 and EpCAM (epithelial cell surface adhesion molecule), among others[109]. Recently, the exosome protein profiles of SW480 and SW620 cell lines were compared to identify metastatic factors and signalling molecules fundamental for tumour progression. A major finding was the selective enrichment of metastatic factors (MET, S100A8, S100A9, TNC) and signal transduction molecules (EFNB2, JAG1, SRC, TNIK) in exosomes derived from the metastatic cells[110].

Animal models

Animal models can be an alternative to clinical samples for the discovery of biomarkers since proteomic studies using inbred strains of mice avoid genetic and physiological variations among individuals. Unfortunately, there are only a small number of mouse models that show genetic alterations that lead to CRC. Among such murine models

are the Apc (Min/+) mice, which are mutant in the *APC* gene and can develop tumours in the small intestine and the colon. The analyses of serum and tumours alterations of the proteins catepsins B and D, DJ-1, clusterin and S100A9 have been described[111]. In another study, among a total of 52 de-regulated proteins identified when comparing tumours and adjacent healthy tissue, a co-expressed gene network linked to innate immunity and inflammation was found in tumours. The network included cathelicidin antimicrobial peptide (CAMP), Toll-like receptors, IL-8 and triggering receptor expressed on myeloid cells 1 (TREM1)[112]. More recently, serum proteins from tumour-bearing Apc (Min/+) mice were quantitatively compared to tumour-free wild type mice *via* in anima metabolic labelling and LTQ Orbitrap mass spectrometer. Among the 40 differentially expressed proteins identified, MGAM, ITIH3 and F5 were validated in neoplastic colonic tissues from the mutant mice. These proteins provided a set of candidate biomarkers for future validation in humans[113].

An overview of recent reports focused on the different proteomic techniques and samples used for the discovery of candidate protein markers for CRC is provided in Table 1.

VALIDATION OF CANDIDATE BIOMARKERS

Many proteomic studies have identified CRC-associated proteins, but little work has been done to validate these candidate biomarkers. Therefore, it would be worthwhile to collect samples from larger cohorts of healthy individuals and CRC patients and to analyse their peptidome and proteome in depth to fully assess the biomarker potential of proteomic changes. Only this validation will then allow transferring novel biomarkers into clinical use for a better detection and treatment of CRC.

Nowadays, antibody-based methods (Western blotting, IHC, microarrays and particularly ELISA) are the most widely used for quantitative biomarker measurement. Nevertheless, antibody specificity is often lacking and considerable care must be taken in the validation of the signals observed. Insufficient samples, often of poor quality, are a key problem, as well as a lack of suitable antibodies or ELISA kits. Additionally, developing a validated ELISA is expensive and time-consuming. This fact, together with the recent advances in MS technology, is further stimulating the development of quantitative MS technologies for protein and peptide biomarker analysis. Multiple reaction monitoring (MRM, also known as selective reaction monitoring, SRM) is rapidly becoming the method of choice[94]. With this technique tens of protein candidates can be followed up in one LC-MS/MS analysis without the use of antibodies, and employing only microliters of sample though it cannot be still performed routinely. Nowadays, MALDI-TOF-MS for peptide profiling acquisition may be also applied as an alternative method for diagnosis of CRC[50]. Moreover, it is expected that combining several markers for CRC and applying multivariate analyses will significantly improve their diagnostic performance[114-116].

Future work should also be directed to evaluate the functional protein interaction networks derived from the proteomics data, in order to elucidate the molecular mechanisms of CRC carcinogenesis. Recently, Jimenez *et al*[83] reported a summary of differently expressed proteins in clinical proteomics studies comparing colorectal healthy and cancer tissues. Authors also analysed these proteins using the STRING tool (http://string.embl.de/) to visualize the protein-protein interaction networks. At the heart of the network they found a cluster of five up-regulated and well-connected proteins in CRC (enolase 1, glyceraldehyde-3-phosphate-dehydrogenase, pyruvate kinase isoenzymes M1/M2, fructose-bisphosphate aldolase A and transketolase), all involved in glycolysis. Glycolytic activity is increased in almost all cancers to provide cells with enough energy for proliferation, a phenomenon called "Warburg effect"[117]. Other molecular and cellular functions associated with the up-regulated proteins include "Cell Death", "Cell-To-Cell Signalling and Interaction" and "Cellular Assembly and Organization"[83].

CONCLUSION

The discovery of novel biomarkers in CRC is crucial for the early detection of the disease, the characterization of the disease progression and the prediction to therapy response. Recent advances in proteomic technologies allow large-scale analysis of proteins which can be applied for discovering aberrant protein profiles of clinical and other biological samples related to CRC. A first critical issue in the search for candidate markers is the selection of the sample set. Tumour tissue is the most direct approach for biomarkers discovery as they are most likely present in cancer tissues at higher concentration than in serum or plasma. In the next step, protein candidate markers must be assessed by a targeted assay and validated in large independent cohorts. Only this validation will allow the translation of the candidate CRC markers to the clinical practice. Finally, it is now generally accepted that a combination of proteins rather than a single individual candidate can better discriminate CRC patients from controls, or determine the prognosis of the patients, implying the advantage of using proteins involved in different physiopathological pathways. In conclusion, there has been a significant amount of research for the identification of CRC biomarkers using proteomic techniques; however, these are yet to yield stronger candidates. A single unique biomarker, of sufficient sensitivity and specificity, may not be a realistic goal, but instead a panel of markers may be a useful way to overcome the difficulties imposed by inter-individual variability.

Table 1 Overview of recent proteomic reports focused on the discovery of candidate protein markers for colorectal cancer

Gene	Protein name	Sample	Potential clinical value	Proteomic technique	Validation	Ref.
YWHAZ	14-3-3 protein zeta/delta	Tissue	Tissue biomarker	2-DE, LC-MS/MS	WB	[24,46]
HLA-A1	HLA class Ⅰ histocompatibility antigen A-1	Membrane fraction of tissue	Tissue biomarker	1-DE, iTRAQ labelling, LC-MS/MS	WB	[105]
ACVR2B	Activin receptor type-2B	Serum	Diagnosis	FP-protein microarray	WB, RP-tissue microarray, ELISA	[78]
PLIN2	Adipophilin	Serum	Diagnosis	Label-free quantitative LC-MS/MS	WB, RP-microarray, IHC	[65]
SPTBN1	Beta-spectrin	Cell lines	Metastasis	1-DE, LC-MS/MS	RNA-Seq	[97]
A1AT	Alpha-1 antitrypsin	Serum, stool	Diagnosis	SELDI-TOF-MS, 1-DE, MudPIT, SRM	-	[69,94]
ORM1	Alpha-1-acid glycoprotein type 1	Serum, stool	Diagnosis	1-DE, MudPIT, SRM	SRM	[77,94]
ORM2	Alpha-1-acid glycoprotein type 2	Serum, stool	Diagnosis	iTRAQ labelling, LC-MS/MS, 1-DE, MudPIT, SRM	WB, ELISA	[73,94]
ENO1	Alpha-enolase	Tissue, validated in serum	Diagnosis	2-DE, 2D-DIGE, LC-MS/MS, MALDI-TOF-MS	WB	[18,19,46]
AFP	Alpha-fetoprotein	Serum	Liver metastasis from CRC	MALDI-TOF-MS, LC-MS/MS	-	[79]
AGT	Angiotensinogen	Serum	Liver metastasis from CRC	MALDI-TOF-MS, LC-MS/MS	-	[79]
ANXA2	Annexin A2	Membrane fractions from colorectal carcinoma	Diagnosis and presumably therapy	2D-DIGE, MALDI-TOF-MS	RP-tissue microarray	[25]
ANXA4	Annexin A4	Membrane fractions from colorectal carcinoma	Diagnosis and presumably therapy	2D-DIGE, MALDI-TOF-MS	RP-tissue microarray	[25,80]
APOA1	Apolipoprotein A1	Serum	Diagnosis	2-DE, 2D-DIGE, MALDI-TOF-MS, LC-MS/MS	WB, RP-protein microarray, RP-tissue microarray	[40,70,80]
CTNNB1	Beta-catenin	Cell lines	Metastasis	1-DE, iTRAQ labelling, LC-MALDI-TOF-MS, LC-MS/MS	WB, RP-tissue microarray, flow cytometry, confocal microscopy	[98,107]
CACYBP	Calcyclin binding protein	Cell lines	Metastasis	iTRAQ labelling, LC-MALDI-TOF-MS	WB	[63,98]
CDH17	Cadherin 17	Membrane fractions from cell lines, exosomes, stool	Metastasis	1-DE, LC-MS/MS	WB, RP-tissue microarray, flow cytometry, confocal microscopy	[91,107,109]
CEACAM5	Carcinoembryonic antigen	Cell lines, membrane fraction from tissue, exosomes, stool	Prognosis	Protein microarray, iTRAQ labelling, 1-DE, LC-MS/MS	WB, RP-tissue microarray, flow cytometry, confocal microscopy, RNA-Seq	[38,91,97,105,107,109]
CAMP	Cathelicidin antimicrobial peptide	Tissue from animal model	Candidate tissue biomarker	2-DE, MudPIT, LC-MS/MS	-	[112]
CTSB	Cathepsin B	Animal model	Candidate diagnosis biomarker	MudPIT, LC-MS/MS	WB, Ab microarray, IHC	[111]
CTSD	Cathepsin D	Tissue, animal model	Candidate diagnosis biomarker	2D-DIGE, MudPIT, LC-MS/MS	WB, Ab microarray, IHC	[80,111]
CDC2	Cell division control protein 2 homolog	Cell lines	Candidate tissue biomarker	2-DE, MALDI-TOF-MS	WB	[95]
CLDN3	Claudin-3	Membrane fraction from tissue, exosomes	Tissue biomarker	1-DE, iTRAQ labelling, LC-MS/MS	WB	[105,108]
PTPRC	Cluster of differentiation 45	Tumor tissue	Diagnosis	Protein microarray	-	[38]
TFRC	Cluster of differentiation 71	Tumor tissue	Diagnosis	Protein microarray	-	[38]
CLU	Clusterin	Serum, animal model	Diagnosis	2-DE, MALDI-TOF-MS, MudPIT, LC-MS/MS	WB, antibody microarray, IHC	[23,70,111]
F5	Coagulation factor Ⅴ	Animal model	Candidate serum biomarker	Metabolic labelling, LC-MALDI-TOF-MS, SRM, LC-MS-MS	-	[113]

COL6A1	Collagen, type Ⅵ α-1 chain	Tissue	Prognosis	LC-MS/MS	WB, IHC	[64]
DPYSL2	Collapsin response mediator protein-2	Secretome from cell lines, validated in serum	Diagnosis	1-DE, MALDI-TOF-MS	WB, IHC, ELISA	[101]
C3	Complement C3	Serum, stool	Diagnosis	SELDI-TOF-MS, 1-DE, MudPIT, SRM, LC-MS/MS	SRM	[69,74,77,94]
C4A	Complement C4-A	Serum	Liver metastasis from CRC	MALDI-TOF MS, LC-MS/MS	-	[79]
C9	Complement C9	Serum	Diagnosis early stage	LC-MS/MS, SRM	RP-protein microarray, SRM	[40,77]
PPIA	Cyclophilin A	Tissue	Tissue biomarker	2-DE, SELDI-TOF-MS	IHC	[58]
CAI	Cytoplasmic carbonic anhydrase Ⅰ	Tissue	Tissue biomarker	2D-DIGE, MALDI-TOF-MS	WB, IHC	[27,33,84,86]
CAII	Cytoplasmic carbonic anhydrase Ⅱ	Tissue	Tissue biomarker	2D-DIGE, MALDI-TOF-MS	WB, IHC	[33]
DEFA1	Defensin-1	Tissue, serum, stool	Diagnosis	SELDI-TOF-MS, MALDI-TOF-MS	-	[68,91]
DEFA2	Defensin-2	Tissue, serum	Diagnosis	SELDI-TOF-MS, MALDI-TOF-MS	-	[68]
DEFA3	Defensin-3	Tissue, serum, stool	Diagnosis	SELDI-TOF-MS, MALDI-TOF-MS	-	[68,91]
DES	Desmin	Tissue, validated in serum	Diagnosis and prognosis	2-DE, MALDI-TOF-MS	WB, IHC	[20]
MAPRE1	The adenomatous polyposis coli-binding protein	Tissue	Prognosis	2D-DIGE	IHC	[32]
CDH1	E-cadherin	Secretome from cell lines , serum	Diagnosis	2D-DIGE, MALDI-TOF-MS, LC-MS/MS	WB	[102,103]
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	Secretome of cultured fresh normal and CRC tissues	Diagnosis	1-DE, LC-MS/MS	WB, IHC	[13]
EFNB2	Ephrin-B2	Exosomes	Metastasis	1-DE, LC-MS/MS	-	[108,110]
EGFR	Epidermal growth factor receptor	Cell lines, exosomes	Candidate diagnosis biomarker	1-DE, LC-MS/MS	WB, RP-tissue microarray, flow cytometry, confocal microscopy, RNA-Seq	[97,107,109]
EPCAM	Epithelial cell surface adhesion molecule	Exosomes	Candidate diagnosis biomarker	LC-MS/MS	-	[109]
GSPT2	Eukaryotic peptide chain release factor GTP-binding subunit	Serum	Liver metastasis from CRC	MALDI-TOF-MS, LC-MS/MS	-	[79]
XPO4	Exportin	Cell lines	Tissue biomarker	1-DE, LC-MS/MS	RNA-Seq	[97]
EZR	Ezrin	Cell lines	Tissue biomarker	1-DE, LC-MS/MS	RNA-Seq	[97]
FABP1	Fatty acid-binding protein 1	Tissue	Prognosis	2D-DIGE, ICAT, LC-MS/MS	WB, IHC	[62]
FGFR4	Fibroblast growth factor receptor 4	Serum	Diagnosis	FP-protein microarray	-	[78]
FGA	Fibrinogen alpha	Serum, stool	Liver metastasis from CRC, diagnosis	MALDI-TOF MS, LC-MS/MS, 1-DE, MudPIT, SRM	-	[79,94]
BLVRB	Flavin reductase	Tissue	Tissue biomarker	2-DE, SELDI-TOF-MS	IHC	[58]
LGALS3	Galectin 3	Cell lines	Tissue biomarker	2-DE, MALDI-TOF-MS	WB	[95]
LGALS4	Galectin 4	Exosomes	Tissue biomarker	1-DE, LC-MS/MS	-	[108]
GSN	Gelsolin	Tissue, serum	Diagnosis	2D-DIGE, MALDI-TOF-MS, SRM, LC-MS/MS	WB, IHC	[19,77]
GSTA1	Glutathione S-transferase alpha-1	Cell lines	Tissue biomarker	2-DE, MALDI-TOF-MS, LC-MS/MS	WB	[95,96]
GPA33	Glycoprotein A33	Exosomes	Candidate diagnosis biomarker	Immunoaffinity capture microbeads and LC-MS/MS	-	[109]
GDF15	Growth/differentiation factor 15	Secretome from cell lines, validated in serum	Metastasis	LC-MS/MS	WB, ELISA	[71]
HBG	Haemoglobin	Stool	Diagnosis	1-DE, MudPIT, SRM	Immunoassay	[93,94]
HSPA9	Heat shock 70 kDa protein 9 (mortalin)	Cell lines	Tissue biomarker	1-DE, LC-MS/MS	RNA-Seq	[97]
HSPB1	Heat shock protein 27	Tissue, validated in serum	Diagnosis	2-DE, LC-MS/MS	-	[18]

HSPD1	Heat shock protein 60	Serum	Diagnosis of late stage CRC, prognosis?	2D-DIGE, MALDI-TOF-MS	WB, ELISA, Tissue microarray	[72]
HNRNPH2	Heterogeneous nuclear ribonucleoprotein H2	Nuclear fraction from tissue	Tissue biomarker	1-DE, MALDI-TOF-MS, LC-MS/MS	-	[104]
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	Tissue, validated in serum	Diagnosis and prognosis	2-DE , MALDI-TOF-MS, SELDI-TOF-MS	IHC	[58,87]
HNRNPH3	Heterogeneous nuclear ribonucleoprotein H3	Cell lines	Tissue biomarker	2-DE, MALDI-TOF-MS	WB	[95]
HABP2	Hyaluronan-binding protein 2	Serum	Diagnosis	SRM, LC-MS/MS	-	[77]
ITLN1	Intelectin-1	Tissue	Prognosis	2D-DIGE, ICAT, LC-MS/MS	WB, IHC	[62]
ITIH3	Inter-alpha-trypsin inhibitor heavy chain 3	Serum from animal model	Serum biomarker	Metabolic labelling, LC-MALDI-TOF-MS, SRM, LC-MS-MS	-	[113]
IL8	Interleukin 8	Animal model	Candidate tissue biomarker	2-DE, MudPIT, LC-MS/MS	-	[112]
JAG1	Jagged 1 protein	Exosomes	Metastasis	1-DE, LC-MS/MS	-	[110]
JUP	Junction plakoglobin	Membrane fraction from cell lines, tissue	Diagnosis, prognosis and metastasis	1-DE, LC-MS/MS	WB, RP-tissue microarray, flow cytometry, confocal microscopy	[64,107]
KRT5	Keratin 5	Tissue, cell lines	Prognosis	LC-MS/MS, 2-DE, iTRAQ labelling, LC-MALDI-TOF-MS	WB, IHC	[64,98]
KNG1	Kininogen-1	Serum, tissue	Diagnosis	MB-WCX, MALDI-TOF-MS	ELISA, IHC	[76]
MIF	Macrophage migration inhibitory factor	Tissue, validated in serum	Diagnosis	2-DE, LC-MS/MS	-	[18]
MGAM	Maltase-glucoamylase	Serum from animal model	Candidate serum biomarker	Metabolic labelling, LC-MALDI-TOF-MS, SRM, LC-MS-MS	-	[113]
MAPKAPK3	MAP kinase-activated protein kinase 3	Serum	Diagnosis	FP-protein microarray	WB, RP-tissue microarray, ELISA	[78]
MET	Met-proto-oncogen	Cell lines, exosomes	Metastasis	1-DE, LC-MS/MS	RNA-Seq	[97,110]
PRTN3	Myeloblastin	Stool	Diagnosis	1-DE, MudPIT, SRM	Immunoassay	[94]
MNDA	Myeloid cell nuclear differentiation antigen	Nuclear fraction from tissue	Tissue biomarker	na1-DE, MALDI-TOF-MS, LC-MS/MS	-	[104]
MYH10	Myosin 10	Cell lines	Candidate tissue biomarker	iTRAQ labelling, LC-MALDI-TOF-MS	WB	[98]
MARCKS	Myristoylated alanine-rich C-kinase substrate	Cell lines	Candidate tissue biomarker	iTRAQ labelling, LC-MALDI-TOF-MS	WB	[98]
NCAM1	Neural cell adhesion molecule 1	Cell lines	Candidate tissue biomarker	iTRAQ labelling, LC-MALDI-TOF-MS	WB	[98]
NNMT	Nicotinamide N-methyltransferase	Tissue, validated in serum	Diagnosis	2-DE, MALDI-TOF-MS	WB, ELISA	[46]
NUP62	Nuclear pore complex 62	Nuclear fraction from tissue	Tissue biomarker	1-DE, MALDI-TOF-MS, LC-MS/MS	-	[104]
NUP88	Nuclear pore complex 88	Nuclear fraction from tissue	Tissue biomarker	1-DE, MALDI-TOF-MS, LC-MS/MS	-	[104]
NPM1	Nucleophosmin	Cell lines	Candidate tissue biomarker	1-DE, LC-MS/MS	-	[97]
NME1	Nucleoside diphosphate kinase A	Tissue, cell lines, serum	Diagnosis	2-DE, 2D-DIGE, RP-tissue microarray, MALDI-TOF-MS, LC-MS/MS	WB, IHC	[4,19,20,24,39,46,84]
NME2	Nucleoside diphosphate kinase B	Tissue	Tissue biomarker	2-DE, SELDI-TOF-MS	IHC	[58]
OLFM4	Olfactomedin-4	Secreted proteins from tissue	Tissue biomarker	OFFGEL, iTRAQ labelling, MALDI-TOF-MS	IHC	[88]
PARK7	Parkinson protein 7	Tissue, animal model	Tissue biomarker	2-DE, LC-MS/MS	WB, IHC	[24,111]
PRDX2	Peroxiredoxins 2	Cell lines	Candidate tissue biomarker	2D-DIGE, MALDI-TOF-MS, LC-MS/MS	WB	[102]
PRDX6	Peroxiredoxins 6	Cell lines	Candidate tissue biomarker	2D-DIGE, MALDI-TOF-MS, LC-MS/MS	WB	[102]
PGK1	Phosphoglycerate kinase Ⅰ	Cell lines	Candidate tissue biomarker	2-DE, MALDI-TOF-MS	WB	[95]
ADCYAP1	Pituitary adenylate cyclase-activating polypeptide	Tissue	Tissue biomarker	2-DE, SELDI-TOF-MS	IHC	[58]

PCNA	Proliferating cell nuclear antigen	Cell lines	Candidate tissue biomarker	2-DE, MALDI-TOF-MS	WB	[95]
PSME3	Proteasome activator complex subunit 3	Tissue, validated in serum	Diagnosis and prognosis	2-DE, MALDI-TOF-MS	IHC	[46,47]
RBBP4	Retinoblastoma binding protein 4	Cell line, tissue	Candidate tissue biomarker	2-DE, LC-MS/MS	WB	[24]
ARHGDI	Rho GDP-dissociation inhibitor	Tissue, cell lines	Metastasis	2-DE, MALDI-TOF-MS		[21]
S100A11	S100-A11 calcium binding protein	Tissue	Tissue biomarker	2-DE, SELDI-TOF-MS	IHC, protein microarray	[58,59]
S100A12	S100-A12 calcium binding protein	Tissue, stool, validated in serum	Diagnosis	2-DE, MALDI-TOF-MS, LC-MS/MS	Immunoassay	[48,93]
S100A6	S100-A6 calcium binding protein	Membrane fraction from tissue	Tissue biomarker	1-DE, 2-DE, MALDI-TOF-MS, LC-MS/MS	-	[81,110]
S100A8	S100-A8 calcium binding protein	Tissue, cell lines, exosomes, validated in serum, stool	Diagnosis	1-DE, 2D-DIGE, MALDI-TOF-MS, LC-MS/MS	WB, IHC	[19,84,91,110]
S100A9	S100-A9 calcium binding protein	Tissue, cell lines, exosomes, animal model, validated in serum, stool	Diagnosis	1-DE, 2D-DIGE, MALDI-TOF-MS, LC-MS/MS	WB, IHC	[19,84,85,91,110]
SELENBP1	Selenium binding protein 1	Tissue, cell lines, serum	Prognosis	2D-DIGE, MALDI-TOF/TOF, and MALDI-TOF-MS	WB	[19,84,86]
PIM1	Serine/threonine-protein kinase Pim-1	Serum	Diagnosis	FP-protein microarray	WB, RP-tissue microarray, ELISA	[78]
SAA2	Serum amyloid A-2 protein	Serum	Diagnosis	1-DE, MudPIT, LC-MS/MS	SRM	[77]
TAGLN	Smooth muscle protein 22-alpha	Tissue	Tissue biomarker	2-DE, SELDI-TOF-MS	IHC	[58]
SPTAN1	Spectrin	Cell lines	Candidate tissue biomarker	1-DE, LC-MS/MS	RNA-seq	[97]
SRC	Proto-oncogene tyrosine-protein kinase Src	Serum, exosomes	Diagnosis, metastasis	FP-protein microarray, 1-DE, LC-MS/MS	WB, RP-tissue microarray, ELISA	[78,110]
STMN1	Stathmin	Cell lines	Metastasis	iTRAQ, LC-MALDI-TOF-MS	WB	[98]
STOML2	Stomatin-like protein 2	Tissue, serum	Diagnosis and prognosis	LC-MS/MS	ELISA	[106]
TNC	Tenascin C	Exosomes	Metastasis	1-DE, LC-MS/MS	-	[110]
TRIP	Thryroid receptor interactor	Serum	Diagnosis	2D-DIGE, MALDI-TOF MS	-	[30]
TIMP1	TIMP metallopeptidase inhibitor 1	Serum	Diagnosis	Lectin Fractionation, MALDI-MS-MS	SRM	[75]
TLRs	Toll-like receptors	Animal model	Candidate tissue biomarker	2-DE, MudPIT, LC-MS/MS	-	[112]
TNIK	TRAF2 and NCK-interacting protein kinase	Exosomes	Metastasis	1-DE, LC-MS/MS	-	[110]
TALDO1	Transaldolase 1	Serum	Diagnosis	2D-DIGE, MALDI-TOF MS	-	[30]
TFRC	Transferrin	Serum, stool	Diagnosis	SELDI-TOF-MS, 1-DE, MudPIT, SRM	-	[69,94]
TGFBI	Transforming growth factor β-induced protein ig-h3	Cell lines	Candidate serum biomarker	2-DE, 2D-DIGE, MALDI-TOF-MS	-	[35,46]
TAGLN	Transgelin	Tissue	Prognosis	2D-DIGE, ICAT, LC-MS/MS	WB, IHC	[62]
TPT1	Translationally controlled tumour protein	Secretome from cell lines, tissue	Candidate serum biomarker	2D-DIGE, MALDI-TOF-MS	-	[35,84]
TTR	Transthyretin	Serum	Metastasis	2-DE, MALDI-TOF MS	ELISA	[11]
TFF3	Trefoil factor 3	Secretome from cell lines, tissue, validated in serum	Metastasis	LC-MS/MS	WB, ELISA	[71]
TREM1	Triggering receptor expressed on myeloid cells 1	Animal model	Candidate tissue biomarker	2-DE, MudPIT, LC-MS/MS	-	[112]
TPM2	Tropomyosin 2 (beta chain)	Tissue	Prognosis	2D-DIGE, ICAT, LC-MS/MS	WB, IHC	[62]
TUBB	Tubulin beta chain	Tissue, cell lines	Diagnosis and prognosis	2DE, iTRAQ labelling, LC-MALDI-TOF-MS, LC-MS/MS,	WB, IHC	[64,98]

PKM	Pyruvate kinase type M2	Tissue, stool	Diagnosis	2D-DIGE, MALDI-TOF-MS	-	[19,92]
VCP	Valosin-containing protein	Tissue	Prognosis	2D-DIGE, ICAT, LC-MS/MS	WB, IHC	[62]
VIL1	Villin 1	Tissue, cell lines	Metastasis	iTRAQ labelling, 2-DE, 2D-DIGE, LC-MALDI-TOF-MS, MALDI-TOF-MS	WB	[19,95,98]
VDAC1	Voltage-dependent anion-selective channel protein 1	Membrane fraction from colorectal carcinoma	Diagnosis and presumably therapy	2D-DIGE, MALDI-TOF MS	RP-tissue microarray	[25]

CRC: Colorectal cancer; 2-DE: Two-dimensional electrophoresis; LC: Liquid chromatography; MS: Mass spectrometry; SELDI-TOF-MS: Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; MudPIT: Multidimensional protein identification technology; SRM: Selected reaction monitoring; ELISA: Enzyme-linked immunosorbent assay; IHC: Immunohistochemistry.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Colorectal cancer screening: 20 years of development and recent progress

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Abstract

Colorectal cancer (CRC) is the second most common cancer in Europe and its incidence is steadily increasing. This trend could be reversed through timely secondary prevention (screening). In the last twenty years, CRC screening programs across Europe have experienced considerable improvements (fecal occult blood testing; transition from opportunistic to population based program settings). The Czech Republic is a typical example of a country with a long history of nationwide CRC screening programs in the face of very high CRC incidence and mortality rates. Each year, approximately 8000 people are diagnosed with CRC and some 4000 die from this malignancy. Twenty years ago, the first pilot studies on CRC screening led to the introduction of the opportunistic Czech National Colorectal Cancer Screening Program in 2000. Originally, this program was based on the guaiac fecal occult blood test (FOBT) offered by general practitioners, followed by colonoscopy in cases of FOBT positivity. The program has continuously evolved, namely with the implementation of immunochemical FOBTs and screening colonoscopy, as well as the involvement of gynecologists. Since the establishment of the Czech CRC Screening Registry in 2006, 2405850 FOBTs have been performed and 104565 preventive colonoscopies recorded within the screening program. The overall program expanded to cover 25.0% of the target population by 2011. However, stagnation in the annual number of performed FOBTs lately has led to switching to the option of a population-based program with personal invitation, which is currently being prepared.

Key words: Colorectal cancer; Population-based screening program; Colonoscopy; Fecal occult blood test

Core tip: The rising incidence rate of colorectal cancer (CRC) puts demands on systematic approaches towards secondary prevention. The National CRC Screening Program in the Czech Republic has been running for more than 13 years. Nowadays, guaiac and immunochemical fecal occult blood tests (FOBT) are used, as well as screening colonoscopy. The quality control system was devised with the introduction of CRC Screening Registry. Since 2006, 104565 preventive colonoscopies have been performed: 89752 FOBT$^+$ colonoscopies (85.8%) and 14813 screening colonoscopies (14.2%). Adenomas were diagnosed in 30515 patients undergoing FOBT$^+$ colonoscopy (34.0%), and in 3719 patients through

screening colonoscopy (25.1%). In all preventive colonoscopies, a total of 4193 cancers were registered.

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INTRODUCTION

Colorectal cancer (CRC) poses serious health risks to the European population, mainly in the Central European region, where it is the second most common cancer as well as the second most common cause of cancer deaths[1]. This is an unfortunate fact knowing that CRC belongs to the group of preventable diseases, if diagnosed early[2]. CRC prevention includes two modalities: screening (early diagnosis of a disease in asymptomatic individuals) and surveillance[3] (long-term follow-up of high-risk individuals). Screening is focused on people aged 50 and over. Age represents a low risk factor for sporadic CRC, that is, carcinoma developing in patients with negative family or personal history of CRC or chronic inflammatory bowel disease; this type of carcinoma accounts for 80% to 95% of all CRC cases[4].

Screening procedures are performed in either one or two steps. One-step programs are represented by screening colonoscopy[5], flexible sigmoidoscopy[6-8], and CT colonography[9]. The initial method of two-step programs involves fecal occult blood tests (FOBT), which can be either guaiac-based (gFOBT)[10,11] or immunochemical (FIT)[12]. In cases of positive FOBT, examination with colonoscopy or flexible sigmoidoscopy follows. Recently, studies with other tests (such as capsule colonoscopy[13,14] or DNA testing[15]) have been performed, but their efficiency in practice has yet to be proved.

Guaiac FOBTs are the most frequently used test in screening programs worldwide. The malignant transformation of premalignant lesions (adenomas) lasts 8-10 years on average[16]. These lesions are often accompanied only by irregular and occult bleeding. However, with repeated and regular FOBT examination, the chances of detecting advanced adenomas or early cancers (followed by successful treatment) are high. It is documented that CRC diagnosis in an asymptomatic individual is associated with a 90% five-year survival rate, but this proportion decreases to 40% and 25% if the symptoms last for 3 or 7 mo, respectively[17]. Similarly, the five-year relative survival[18] can range between 90% and 15% for localized and advanced cancers, respectively[19].

The key point of the screening programs is to reach adequate target population coverage. Therefore, an organized population-based screening program based on early identification and followed by personal invitation to each individual from the target population is preferred[20].

CANCER BURDEN IN CENTRAL EUROPE

In Europe in 2012, it is estimated that 3.45 million new cases of cancer were diagnosed and 1.75 million patients died from malignant diseases. Concerning CRC, the annual number stands at 447000 new cases, with 215000 fatalities[21]. The burden of CRC is not equally distributed across Europe. Central European countries, most notably Slovakia, Hungary, and the Czech Republic, rank among the countries with the highest CRC incidence and mortality rates in Europe, with values two to three times higher than countries with the lowest occurrence (*e.g.*, Bosnia and Herzegovina, Greece, and Albania). The mortality to incidence (MI) ratio has been shown to be a good indicator of cancer-specific survival[22]. With a CRC MI ratio of 0.42, the Czech Republic is close to the EU-27 value (0.40). Within Central Europe, similar results were shown for Austria (0.41) and Slovakia (0.44).

Time trends in CRC incidence in the Czech Republic and neighboring countries show diverse patterns for the last two decades (Figure 1, Table 1; selected cancer registries with trends available over long periods of time were chosen to represent Germany and Poland). Whereas in 1990, Saarland in Germany was the area with the highest CRC incidence, followed by the Czech Republic and Austria, in 2000, the Czech CRC incidence rates ranked first, representing a 25% increase in incidence over a decade. An even sharper increase was seen in Slovakia (+33%) and in Kielce, Poland (+80%). Fortunately, these trends were not repeated during the most recent period: CRC incidence rates have decreased (Czech Republic, Germany, and Austria) or only moderately increased (Slovakia, Poland).

In the Czech Republic, 8265 new CRC cases were diagnosed in 2010 with 3991 deaths. CRC incidence has been increasing alarmingly from the start of cancer registration in the 1970s up to the early 2000s. Recently, CRC incidence dropped by 4.4% between the periods 1995-1999 and 2006-2010. An even more substantial decrease was observed in CRC mortality rates, which dropped by 20.8% between 1995-1999 and 2006-2010 (Figure 2). In 2010, 23.8% of the patients were diagnosed with stage 3 and 23.0% in the primary metastatic stage (Figure 3). However, the first positive trends in early diagnosis have been witnessed; whereas only 15.6% of patients were diagnosed with stage 1 CRC in 2000, this proportion increased to 23.3% in 2010.

The epidemiological situation has been a challenge in the designing of a more effective CRC screening program in the Czech Republic. The program has been developed in step by step phases.

CZECH NATIONAL CRC SCREENING PROGRAM

CRC screening pilot studies

Due to the unfavorable development of CRC incidence and mortality rates in the Czech Republic in the second

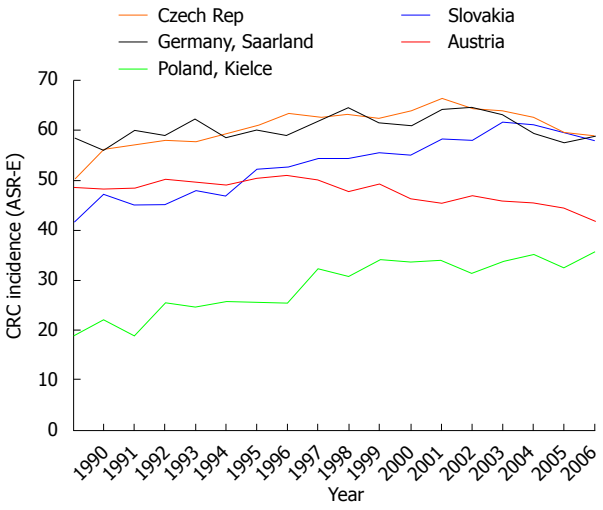

Figure 1 Time trends for incidence rates of colorectal cancer in the Czech Republic and neighboring countries. CRC: Colorectal cancer; ASR-E: Number per 100000 population, age-standardized rate (European).

half of the 20th century, six pilot studies incorporating gFOBT were performed in the period 1979-1984, and their summary results were published in 1986[23].

These studies were followed by the subsequent phase, referred to as the "Czech Screening Program", in the period 1985-1991. This program was conducted in all regions and involved more than 109000 asymptomatic individuals between 45 and 60 years. Compliance of the target population reached 83.1%. Cancers diagnosed within this screening program were detected in earlier stages than non-screening cancers[24].

To confirm these outcomes in a different political and medical care setting (national *vs* private health care), yet another prospective study was conducted under the label "The Prague Project" (1997-1998). Here, 68 general practitioners (GPs) were involved and 12600 asymptomatic individuals were examined, with 80% compliance.

Both studies confirmed favorable cost-benefit and cost-effectiveness results, interest of the target population, as well as readiness of health care professionals to support CRC screening.

Introduction to the national program

Based on these facts, negotiations among the Czech Society of Gastroenterology, the General Health Insurance Company, and the Czech Ministry of Health followed, leading to the introduction of the National CRC Screening Program on July 2000. At that time, the Czech Republic was only the second country to have a nationwide CRC screening program (with Germany being the first). In the two-step program, the biennial guaiac FOBT (three stool samples) was offered to asymptomatic individuals aged over 50 as part of preventive check-ups at GP clinics[25]. In cases of a positive test, colonoscopy followed.

Further development

The two phases in the developmental course of the program can be distinguished. In the period 2000-2005, the program was implemented and established; from 2006 until now, it was continuously improved and evolved. Compared to the other widely recommended screening programs (focused on breast cancer and cervical cancer), the CRC program is a multidisciplinary issue. In the beginning, the organizational structure comprising of GPs and gastroenterologists was established. Over the first three years, specific financial support (approximately CZK 240 million, equivalent to EUR 9 million) was allocated, with the objective of substantial improvements in the equipment of endoscopy units. A media campaign was launched, and educational courses were held in all regions. The program has been monitored and evaluated by the CRC Screening Council, consisting of regional coordinators of all involved medical specialties, and by the CRC Screening Committee of the Czech Ministry of Health. Until 2005, the evaluation was only based on aggregated data (provided by health insurance companies). During this period, 977973 gFOBTs were performed, 19257 adenomas were removed, and 2797 cases of CRC were diagnosed.

Table 1 Time trends for incidence rates for colorectal cancer in the Czech Republic and neighboring countries

Cancer registry	Colorectal cancer incidence-ASR (E)				
	1990	2000	Trend 1990-2000	2007	Trend 2000-2007
Czech Republic	50.3	62.7	+25%	59.0	-6%
Germany, Saarland	58. 8	61.7	+5%	58.7	-5%
Slovakia	41.7	55.7	+33%	57.9	+4%
Austria	48.8	49.4	+1%	41.8	-16%
Poland, Kielce	19.1	34.3	+80%	35.7	+4%

Source of data: Steliarova-Foucher *et al*[21] ECO; Czech National Cancer Registry. ASR (E): Number per 100000 population, age-standardized rate (European).

Assessment of quality control for program improvement

In 2006, the Czech CRC Screening Registry for collecting anonymous individual data was established. This online database contains data from the nationwide network of high-quality endoscopy units (168 centers for screening colonoscopy) on all preventive colonoscopies. The term "preventive colonoscopy" covers both FOBT+ colonoscopies (performed after a positive FOBT) and screening colonoscopies (available to all individuals aged over 55 years). The centers are required to meet strict quality criteria, including adequate personnel, materials and equipment, the recommended annual volume of colonoscopies and endoscopic polypectomies, quality control system, and a plan for the management of complications. The registry includes demographic data, the type and date of FOBT applied, and the main findings of the performed colonoscopy. Records in the registry also involve information about the number, size and histology of adenomas, the preoperative staging and histology of cancers, as well as complications (severe bleeding, perforation) during diagnostic procedures and polypectomies. The collected data is centrally stored and analyzed at the

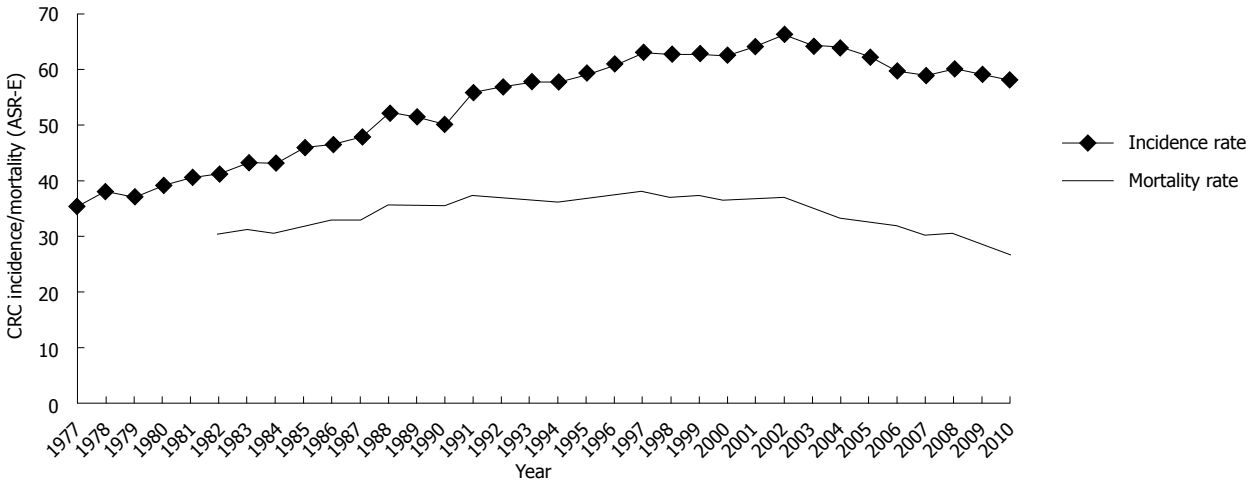

Figure 2 Time trends for the incidence and mortality rates for colorectal cancer in the Czech Republic. CRC: Colorectal cancer; ASR-E: Number per 100000 population, age-standardized rate (European).

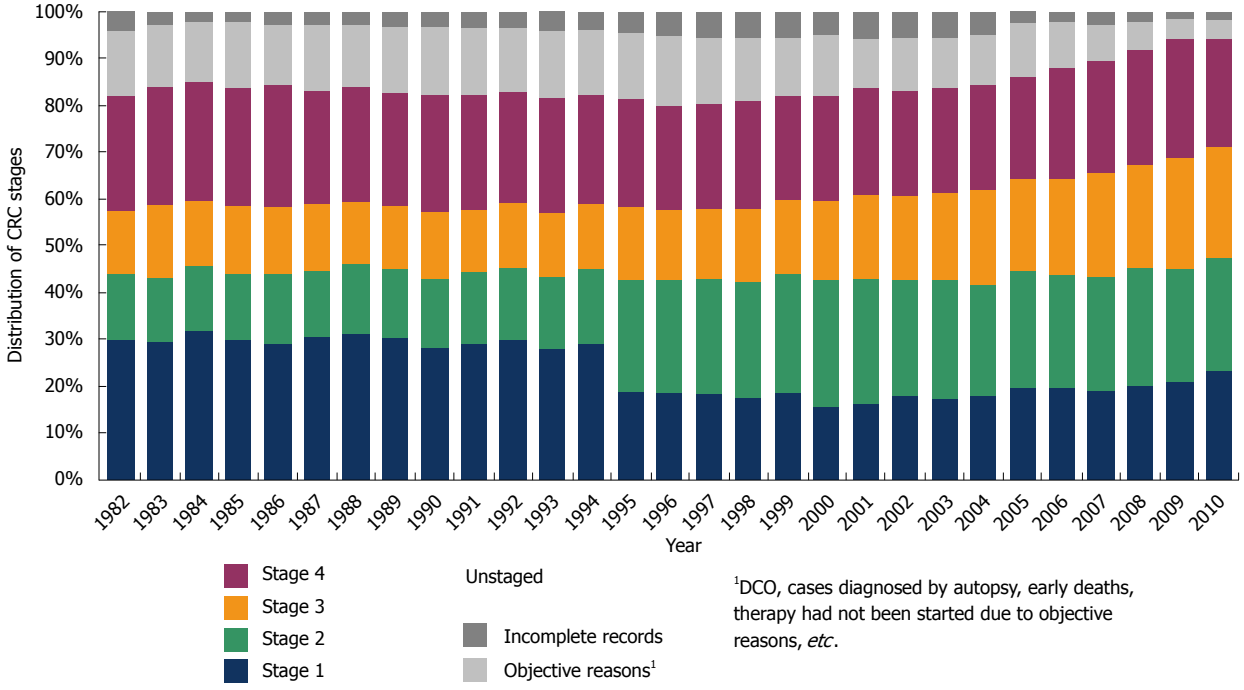

Figure 3 Time trends for the distribution of stages in newly diagnosed colorectal cancer patients in the Czech Republic. CRC: Colorectal cancer.

Institute of Biostatistics and Analyses of Masaryk University, Brno). The results of the program are available at the national website: www.kolorektum.cz.

Data entered in the registry is used for quality control of the program, with the main indicators as defined by the European guidelines[26,27]. The indicators are: adenoma detection rate (ADR), advanced ADR, cecal intubation rate, positive predictive value, and endoscopic complications[28].

Since 2006, a total of 104565 preventive colonoscopies have been performed: 89752 FOBT$^+$ colonoscopies (85.8%) and 14813 screening colonoscopies (14.2%). Adenomas were diagnosed in 30515 patients undergoing FOBT$^+$ colonoscopy (34.0%; 48.1% of them with advanced adenomas) and in 3719 patients undergoing screening colonoscopy (25.1%; 29.4% of them with advanced adenomas). In all preventive colonoscopies, a total of 4,193 cancers were registered. The overall cecal intubation rate reached 94.7% in FOBT$^+$ colonoscopies and 97.7% in screening colonoscopies. In the years 2006-2012, there were 92 cases of perforation (0.09% of all examinations) and 361 cases of bleeding during endoscopic polypectomies (0.76% of all therapeutic procedures) reported.

The Czech National Cancer Registry (CNCR) and the Czech National Reference Centre (CNRC) are additional sources for monitoring the quality of the screening program. The Czech National Cancer Registry, the data of which was made partly accessible *via* the www.svod.cz portal[29], is an essential source of cancer statistics data, covering the entire population of cancer patients

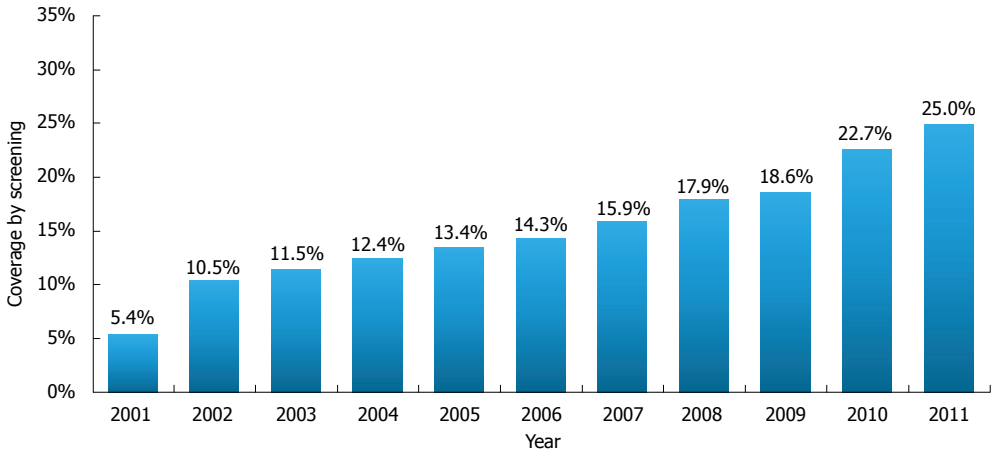

Figure 4 Time trends for coverage by colorectal cancer screening (fecal occult blood test and screening colonoscopy) in the Czech Republic.

in the Czech Republic starting from 1977. CNCR is a population registry involving the following information: personal data, tumor diagnosis, treatment, and post-treatment follow-up entries. The population-based monitoring allows us to estimate the incidence and the mortality rates, distribution of the clinical stages, as well as cancer treatment and survival rates. Malignant neoplasms are recorded in accordance with the International Classification of Diseases for Oncology (ICD-O, 10th revision)[30]. Tumor staging is performed on the basis of the TNM classification system[31]. Standardized Death Certificates (internationally recommended by the WHO) are implemented to collect precise individual data on the cause of death[32].

In the Czech Republic, all residents are covered by public health insurance. The Czech National Reference Centre aggregates complete information from all health insurance companies. As the insurance cover is practically universal, this data can be used to estimate the number of preventive and diagnostic colonoscopies, as well as the performed FOBTs. This information system is, therefore, an essential tool for monitoring early performance indicators within the Czech program. In the period 2006-2011, 2405850 FOBTs were performed, with an estimated overall positive result of 5.1%; however, this figure showed an upward trend until it reached 6.7% in 2011.

Current design

The CNRC data shows that the coverage of the target population (all individuals aged over 50) has been steadily increasing since the program's introduction; however, this rise has been very slow and the coverage has been well under the target values as recommended by the European Guidelines (Figure 4). Therefore, in 2009, a new program design was launched with the implementation of FIT, screening colonoscopy, and the involvement of gynecologists. Currently, the program is offered to asymptomatic individuals aged 50 and over. As regards people aged between 50 and 54 years, annual FOBT is offered followed by FOBT^{+} colonoscopy in cases of a FOBT positive result. People aged 55 years and over can choose between FOBT (biannually) and screening colonoscopy (at 10-year intervals). Since early 2013, gFOBT has been phased out. All types of FIT are allowed, both qualitative and quantitative, without determining a uniform cut-off value. Participation of gynecologists in the screening program was beneficial, mainly due to the rise in FIT use. In 2011, 8% of FOBTs were performed by gynecologists; helping to increase the coverage notably in women aged less than 65.

Impact on long-term indicators

Twelve years after its initiation, the Czech CRC screening program now extends coverage to about one in four of the target population. As it is far below the levels recommended by the European Guidelines, a significant impact of the program on CRC incidence and mortality in the target population cannot be expected. However, our data suggests a decrease in CRC incidence over the last decade: any slight decrease in the incidence is accompanied by a substantial fall in mortality and some increase in the proportion of the early stages. It is likely that the advent of CRC screening, combined with improvements in both the quality and capacity of endoscopy centers and increased CRC awareness, has had a positive impact on early diagnosis of CRC. Bearing in mind the slow spread of the screening cover, together with the sluggish natural progression of the adenoma to carcinoma, the impact of screening on CRC incidence is probably rather small, but may become more relevant in the years to come. The observed decrease in CRC mortality rates is the likely effect of both early diagnosis and a more successful treatment regime for CRC, as demonstrated by improvements in stage-specific CRC survival[33].

DISCUSSION

In the last twenty years, CRC screening programs across Europe went through considerable changes[34-37]. The first country to implement an organized program was Germany in 1976, followed by the Czech Republic in the year 2000[38,39].

Table 2 Characteristics of selected fecal occult blood test colorectal cancer screening projects including personal invitations

Country	Age group (yr), test	Time period	Procedure in personal invitation	Participation rate	Source
Studies and pilot projects					
England	50-69 gFOBT	2000-2004	Centralized invitations by the screening unit Sending fecal occult blood test (FOBT) test kits	F: 59% SM: 48% SW: 56%	Weller *et al*[67], 2007 UKCRCSPG[66], 2004
France	50-74 gFOBT	2003-2006	Centralized invitations according to sickness fund database files Invitation to general practitioners (GP), reminder after 6 mo, FOBT kit reminder 4 mo later Exclusions by GP: serious illness, recent colorectal cancer (CRC) screening, high CRC risk	FM: 54% FW: 57%	Denis *et al*[59], 2007
Netherlands	50-74 gFOBT, FIT	2006-2007	Centralized invitations Exclusions: Inflammatory bowel disease (IBD), CRC, recent CRC screening Pre-invitation, sending test kits after 2 wk, reminder after 6 wk	gFOBT: 50% FIT: 62%	Hol *et al*[60], 2010
Scotland	50-69 gFOBT	2000-2007	Centralized invitations by the screening center Sending FOBT test kits Reminder-second kit-after 6 wk (kit in first round only)	FM: 50%, FW: 60% SM: 49%, SW: 57%	Steele *et al*[65], 2009
Spain	50-69 gFOBT	Since 2000	Centralized invitations Exclusions: CRC, adenoma, high CRC risk Invitation-reply-sending test kit, reminder after 6 wk	FM: 17%, FW: 18% SM: 21%, SW: 24%	Peris *et al*[64], 2007
Population-based screening programs					
England	60-69 gFOBT	Since 2006	Centralized invitations by the screening center People registered with a GP practice Sending FOBT test kits	M: 50% W: 54%	Logan *et al*[61], 2012
Finland	60-69 gFOBT	Since 2004	Centralized invitations by the national screening center using Population Register Center Sending FOBT test kits	FM: 62%, FW: 77% SM: 68%, SW: 80%	Malila *et al*[48], 2011 Malila *et al*[63], 2008 Malila *et al*[62], 2005

F: First round of screening; S: Second round of screening; M: Men; W: Women; gFOBT: Guaiac fecal occult blood test; FIT: Fecal immunochemical test.

The development can be attributed to two main processes: fecal occult blood testing evolution and the transition from an opportunistic to population-based program setting. Initially, gFOBTs were used widely, mostly because of the favorable results of a randomized controlled study in 1993 that confirmed its 15%-33% CRC mortality reduction[40], low test cost, and easy handling. In the last decade, many trials showing the superiority of FIT were published[41,42]. Higher sensitivity for colorectal neoplasia and higher target population compliance were detected[43,44]. Some European countries (Great Britain, the Czech Republic, and Germany) have been replacing gFOBT by FIT; some started with FIT from the beginning (the Netherlands and Slovenia)[45]. The main issue is to find an appropriate cut-off level to balance the sensitivity and cost-effectiveness[46]. Most studies prefer the cut-off level in the range of 75-100 ng/mL[47]. In Finland, on the other hand, there is long tradition of using gFOBT, which has not changed over time, mainly because of the favorable results. The participation rate of 80% in women has been achieved for the second round[48]. Poland remains the only country using colonoscopy in organized CRC screening program as the only screening modality[49,50]. Colonoscopy is considered as a gold standard for CRC screening, but there has not been any randomized controlled trial confirming a reduction in CRC mortality by using this method. Therefore, an extensive international study (the NordICC Study) has started to prove this fact from a long-term perspective[51]. In contrast, recent data from England points to a 43% reduction in CRC mortality with flexible sigmoidoscopy screening[52,53]. Outside FOBT, colonoscopy, and flexible sigmoidoscopy, other methods have not yet been implemented as a regular part of screening programs as they are still under development (CT colonography[54], capsule colonoscopy[55], and molecular tests[56,57]).

In 2010, the European Guidelines for Quality Assurance in Colorectal Cancer Screening and Diagnosis were published[58] based on the recent and evidence-based data focused on CRC secondary prevention and diagnosis. Concerning the program organization, it favors the population-based setting that can lead to adequate target population compliance (acceptable level of 45%, recommended level of 65%). Programs including personal invitations were successfully tested or implemented in many European countries and generally achieved very promising results regarding the participation rate of the target population (Table 2)[59-67].

The above developmental steps have been reflected in the colorectal cancer screening program in the Czech Republic. Based on the recent data, with a stagnating annual number of FOBTs, preventive colonoscopies, and diagnosed adenomas and cancers, it seems that the maximum limit of opportunistic screening has been reached (Table 3). A switch to a population-based program is therefore essential and currently being prepared. This nationwide project (run by the Czech Ministry of Health) includes all three national screening programs (colorectal cancer,

Table 3 Basic characteristics of preventive colonoscopies performed within the Czech colorectal cancer screening program, 2006-2012

Basic characteristics	Year	Patients with CS	Patients with detected adenoma	Proportion[1]	Patients with detected CRC	Proportion[1]
FOBT[+] CS	2006	5334	1578	29.6%	335	6.3%
	2007	5679	1635	28.8%	337	5.9%
	2008	7457	2367	31.7%	446	6.0%
	2009	11712	3778	32.3%	599	5.1%
	2010	18327	6235	34.0%	829	4.5%
	2011	20131	7134	35.4%	733	3.6%
	2012	21112	7788	36.9%	770	3.6%
	Total	89752	30515	34.0%	4049	4.5%
Screening CS	2009	1362	345	25.3%	24	1.8%
	2010	4400	1076	24.5%	43	1.0%
	2011	4571	1160	25.4%	42	0.9%
	2012	4480	1138	25.4%	35	0.8%
	Total	14813	3719	25.1%	144	1.0%
Total		104565	34234	32.7%	4193	4.0%

Source: Czech colorectal cancer (CRC) Screening Registry. [1]Proportions of detected neoplasia represent the positive predictive value for fecal occult blood test (FOBT) + colonoscopies (CS) and the detection rate for screening CS.

breast cancer, and cervical cancer). It is based on an organized personal invitation of the eligible population sent to individuals by health insurance companies. Another topic of discussion focusing on further improvements in the program effectiveness includes the choice of a particular FOBT. Immunochemical methods currently used in the screening program differ in their analytical performance. The validation and selection of methods with an appropriate cut-off and quality control may be essential in the future. In a Czech initial study, the optimum cut-off level of quantitative FIT was determined as 75 ng/mL using one test[68]. However, further studies will be necessary for the implementation of an appropriate cut-off to the screening program.

Currently, the uniform design of the CRC screening program is used for asymptomatic individuals, but increased CRC risk has been proven in patients with cardiovascular diseases and diabetes mellitus, type II [69-71]. Therefore, an extensive Czech nationwide study is underway, focusing on the determination and stratification of metabolic risks in the development of colorectal neoplasia, and setting the specific intervals of CRC screening programs for these patients.

National screening programs may significantly improve the current state of public health. They require cooperation among health professionals, the Ministry of Health, and health insurance companies. In contrast to other national cancer prevention programs for breast and cervical cancers, the CRC screening program is aimed at both sexes, although women are statistically more likely to recognize the importance of the screening procedures. CRC screening is more complicated and partially invasive (colonoscopy). The publicity of the program has to be permanent, focusing on the entire population as well as on health professionals. It also needs the necessary support of prominent representatives of various professions from different segments of society. Since the beginning of this century, international scientific societies have drawn attention to the high CRC incidence and mortality rates prevalent in all developed European countries. In 2010, the European Society of Digestive Oncology, the International Digestive Cancer Alliance, the International Agency for Research on Cancer, the United European Gastroenterology, and the Munich Gastroenterology Foundation emphasized in the Barcelona Declaration that CRC is the second most common cause of cancer death. In the EU Parliament, a group of representatives headed by the Czech diplomat Pavel Poc opened a discussion on this topic, and the assembly finally approved the Written Declaration on Fighting Colorectal Cancer in the European Union. Therefore, CRC prevention is not only a medical topic; it is an issue of social policy as well. This fact should be reflected in CRC prevention at all levels, from the general public to the responsible authorities.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Chemopreventive drugs: Mechanisms *via* inhibition of cancer stem cells in colorectal cancer

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Abstract

Recent epidemiological studies, basic research and clinical trials on colorectal cancer (CRC) prevention have helped identify candidates for effective chemopreventive drugs. However, because of the conflicting results of clinical trials or side effects, the effective use of chemopreventive drugs has not been generalized, except for patients with a high-risk for developing hereditary CRC. Advances in genetic and molecular technologies have highlighted the greater complexity of carcinogenesis, especially the heterogeneity of tumors. We need to target cells and processes that are critical to carcinogenesis for chemoprevention and treatment of advanced cancer. Recent research has shown that intestinal stem cells may serve an important role in tumor initiation and formation of cancer stem cells. Moreover, studies have shown that the tumor microenvironment may play additional roles in dedifferentiation, to enable tumor cells to take on stem cell features and promote the formation of tumorigenic stem cells. Therefore, early tumorigenic changes of stem cells and signals for dedifferentiation may be good targets for chemoprevention. In this review, I focus on cancer stem cells in colorectal carcinogenesis and the effect of major chemopreventive drugs on stem cell-related pathways.

Key words: Colorectal cancer; Chemoprevention; Cancer stem cell; Carcinogenesis; Microenvironment

Core tip: To develop optimal chemopreventive agents, we need to target cells and pathways that are essential and critical to carcinogenesis: early tumorigenic changes of stem cells and signals for dedifferentiation may be good targets for chemoprevention. Major chemopreventive drugs, such as non-steroidal anti-inflammatory drugs, statins, proliferator-activated receptor γ agonists and metformin, have cancer stem cell (CSC)-suppressing effects *via* regulation of stem cell-regulating pathways, stem cell niche in the tumor microenvironment, and altered tumor metabolism. These stem cell-related steps in tumorigenesis could be critical targets for chemoprevention and CSC-targeted adjunctive treatment of colorectal cancer.

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CHEMOPREVENTIVE DRUGS FOR COLORECTAL CANCER AND FUTURE DIRECTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers, and is a major cause of cancer morbidity and mortality worldwide[1]. Although there have been improvements in surgical and oncological therapies, the data have shown limited survival improvements in

advanced CRC[2]. Therefore, prevention strategies remain the most promising avenue for reducing both the incidence and mortality of CRC.

CRC development is a multi-step process that occurs over a span of about 10 years, thereby providing an opportunity for prevention and early detection[3]. CRC screening and polyp removal are effective interventions for CRC prevention[3,4]. However, along with screening efforts, we need a specific prevention strategy for patients at high-risk for developing CRC. Chemoprevention involves the use of a variety of agents that can prevent, delay, or even reverse the development of pre-malignant lesions by suppressing the multi-step carcinogenic process. Many studies have demonstrated that pre-malignant lesions can be reversed and prevented pharmacologically[5]. This effect is of particular importance to high-risk individuals with a hereditary predisposition for or susceptibility to the environmental causes of CRC. Chemoprevention shows great promise in this regard and the ideal chemopreventive agent, with an excellent safety profile, remains to be discovered.

Until now, there have been several major candidates for CRC chemopreventive drugs, including aspirin and non-steroidal anti-inflammatory drugs (NSAIDs), statins, peroxisome proliferator-activated receptor (PPAR) γ agonist, and metformin, which exhibit chemopreventive effects in epidemiologic studies, in *in vitro* and *in vivo* experiments, and in some clinical trials.

NSAIDs have received the most attention as chemoprevention agents in CRC, and experimental and clinical studies have consistently shown that NSAIDs may reduce the risk of colorectal adenoma or cancer[6,7]. In experimental models, either nonselective or cyclooxygenase-2 (COX2)-selective NSAIDs have been shown to suppress CRC growth through COX2-dependent and -independent mechanisms, such as activation of apoptotic and anti-inflammatory signals[8,9].

Many clinical trials have addressed the cancer-preventive effect of aspirin, using colorectal adenomas as a surrogate primary end point for cancer, and the data support its benefits in reducing the risk of CRC. In patients with a history of a previous CRC[10] or a history of colorectal adenomas[6,11], the recurrence of adenoma was reduced in patients who received aspirin *vs* those who did not. In addition, in patients with hereditary non-polyposis CRC, the long-term use of aspirin reduced the incidence of CRC, with an HR of 0.63 (95%CI: 0.35-1.13)[12].

In addition to aspirin, other NSAIDs have also shown efficacy in CRC prevention trials. For example, in one clinical trial, in which patients with a history of resected adenomas were randomized to receive either sulindac plus difluoromethylornithine or matched placebos, promising results were seen, in that the risk ratio was 0.30 (95%CI: 0.18-0.49) for recurrent adenomas and 0.085 (95%CI: 0.011-0.650) for advanced adenomas in the intervention arm relative to the placebo arm[13]. In addition, recent long-term follow-up studies have reported that NSAIDs may also reduce the recurrence and mortality of CRC[14-16]. Meanwhile, celecoxib, a selective COX2 inhibitor, showed promise in inhibiting adenoma occurrence in familial adenomatous polyposis patients[17] and in patients with a history of colorectal polyps[18,19]. However, COX2 selective inhibitors are no longer considered for prevention of CRC because of their cardiovascular toxicities[20-22].

Statins, widely used cholesterol-lowering drugs, inhibit cholesterol synthesis *via* inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, the rate-limiting enzyme in the mevalonate and cholesterol-synthesis pathway. Many of the downstream products of this pathway are required for critical cellular functions, such as maintenance of membrane integrity, signaling, protein synthesis and cell cycle progression[23,24]. However, clinical studies examining the relationship between statins and CRC incidence have yielded mixed results. Although some case-control and cohort studies and a meta-analysis study[25-31] demonstrated a protective effect against CRC in statin users, other studies failed to do so[32-37]. Siddiqui *et al*[38] showed reduced recurrence of polyps (OR = 0.51, 95%CI: 0.43-0.60) and high-risk polyps (OR = 0.74, 95%CI: 0.52-0.96), and diminished polyp size and number in patients who used statins continuously for up to 5 years. Moreover, statins have an excellent safety profile. To overcome the possible discrepancies in the results from studies of statins in CRC chemoprevention, a thorough analysis of the underlying CRC risk, methodologies and exposure time are needed, along with data from well-designed large-scale clinical trials and epidemiological studies in the future. The exact role of statins in chemoprevention remains to be elucidated. In addition, recent data have suggested that statins may have beneficial effects on disease progression and survival, indicating that long-term use of statins may be associated with a less-advanced tumor stage and a better survival rate[34,39].

PPARγ agonists, such as thiazolidinedione (TZD), an insulin-sensitizing diabetes drug, also have anti-cancer activities, involving inhibition of cell growth and induction of apoptosis and terminal cellular differentiation[40-43]. PPARs have central roles in the regulation of glucose and lipid homeostasis, and also regulate cell proliferation, differentiation, and inflammation[44]. Recently, several studies have reported that the use of TZDs may be associated with a decreased risk of CRC in patients with diabetes[45,46], and in some cases, PPARγ agonists have also shown modest efficacy for chemoprevention in clinical trials[47,48]. In addition, PPARγ expression in CRC primary tumors correlates well with overall survival of CRC patients[49], which is consistent with animal experiments showing that intestinal tumors are exacerbated in *APC* min/+ mice with genetic ablation of *Pparg*, compared with control *APC* min/+ mice[50]. However, controversy regarding the anti-tumor effect of PPARγ agonists persists because some studies indicated that activation of PPARγ promotes tumorigenesis[51-54]. Furthermore, clinical studies show that TZD may be associated with an increased risk of heart failure[55], bone fractures[56-58] and

possibly bladder cancer[59]. Whether these are PPARγ-mediated side effects or off-target effects remain uncertain. Although PPARγ is currently considered a potential target for chemoprevention, previous results are based mainly on observational and preclinical studies, and rigorous clinical trials are needed to address the utility of PPARγ agonists in CRC chemoprevention.

Metformin is a classic biguanide drug that has been used as first-line therapy for type 2 diabetes mellitus (DM). Metformin inhibits hepatic gluconeogenesis and reduces insulin resistance. It is a safe and economical drug that has been used for more than 50 years. Most CRC-specific observational studies and meta-analyses reported that patients with type 2 DM who were taking metformin have a lower risk of CRC and better outcomes compared with patients not taking the drug[60-64]. Moreover, metformin showed a protective effect for colorectal adenoma recurrence in colonoscopic surveillance of CRC patients with diabetes[65]. Preclinical studies in animal models support these findings, showing that metformin induced AMP-activated protein kinase (AMPK) activation and inhibited tumor development and growth, including colon tumorigenesis[66,67]. In terms of its molecular mechanism, metformin regulates insulin/insulin-like growth factor-related pathways, inflammatory activity, and the AMPK/mammalian target of the rapamycin (mTOR) pathway[68]. Activated AMPK inhibits the mTOR-mediated synthesis of key proteins responsible for malignancy and growth of cancer cells[69], and is thought to be a main mediator of the potential anti-cancer mechanism of metformin. Despite the promising results in these studies, data pertaining to metformin from well-designed clinical trials for CRC and its precancerous lesions are lacking. Furthermore, the anti-tumor effect of metformin on non-diabetic patients should also be assessed, because the safety of metformin is well known and it has no glucose-lowering effects in non-diabetic patients. One clinical trial demonstrated an inhibitory effect of metformin in aberrant crypt foci formation of the rectum in patients who did not have diabetes[70]. However, this study showed a short-term effect of metformin in a small number of subjects. Therefore, large-scale randomized controlled trials are required to confirm the chemopreventive and therapeutic effects of metformin, especially for non-diabetic patients[71].

Among the most-promising chemopreventive drugs currently being studied, NSAIDs have consistently shown a protective effect against CRC; however, they are generally not recommended for widespread chemoprevention because of the increased risk of bleeding[72]. In addition, COX2-selective inhibitors showed increased cardiovascular morbidity[73]. However, statins, PPARγ agonists and metformin have a relatively good safety profile, and these drugs show similarities in their abilities to improve metabolic disorders that are known to be associated with increased cancer risk, such as diabetes, obesity, dyslipidemias, and chronic inflammation. For widespread acceptance of these chemopreventive drugs, more definite chemopreventive effects in large-scale well-designed clinical trials need to be demonstrated, along with a more acceptable safety profile for PPARγ agonists.

Future directions in CRC chemoprevention will include genetic and molecular approaches for identifying pathways that are associated with cancer initiation and development, and personalized approaches to predict risk, drug susceptibility and toxicity. In addition, mechanism-based combinations of agents will also maximize effectiveness, while limiting drug toxicity. From the perspective of identifying new targets for chemoprevention, besides the traditional targeting of the multi-step process in colorectal carcinogenesis, new evidence has demonstrated that targeting essential cell types and critical signaling pathways, such as tumor-initiating stem cells and stem cell-related pathways, could be another effective strategy for preventing colorectal tumorigenesis.

STEM CELLS IN CRC AND EARLY CARCINOGENESIS: OPPORTUNITIES FOR CHEMOPREVENTION

Much evidence has shown that genomic instability, including chromosomal and microsatellite instability, and epigenetic changes are important mechanisms for multistep tumorigenesis of CRC[74]. However, we have come to understand that tumors show inter- and intra-tumoral heterogeneity, even in the same patient, and more complex intercellular interactions. Therefore, we need to identify the cells and cellular interactions that are critical in tumors to eradicate cancer cells and prevent cancer development. Recent evidence revealed that stem cells, the tumor microenvironment and metabolic alterations are closely related and critical steps in colorectal carcinogenesis. With recent advances in our understanding of the homeostatic control of intestinal crypts and microenvironments (niches), we are able to delineate the steps in early carcinogenesis of CRC, which could lead to development of new targets for chemoprevention of CRC.

Within the crypts of the intestinal mucosa, the intestinal epithelium is a permanently renewing tissue, the architecture of which is maintained by the ability of the intestinal stem cells to self-renew and generate a hierarchy of proliferative and differentiated cells[75]. The balance between proliferation and cell death is important for homeostasis of the intestinal epithelium. Using genetic lineage-tracing methods for stem cell markers, several markers of intestinal stem cells, including B-lymphoma Mo-MLV insertion region 1 (BMI1), telomerase reverse transcriptase, leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5), leucine-rich repeats and immunoglobulin-like domains 1 (Lrig1), olfactomedin 4 and achaete-scute complex homolog 2 (ASCL2), have been identified[76-80].

As for the stem cell niche or microenvironment, subepithelial mesenchymal cells, their secreted growth factors, and basement membrane factors that regulate

epithelial cell function support the intestinal crypts. Pericryptal myofibroblasts (PCMFs), one of the microenvironment or niche components, have crucial functions and roles in intestinal organogenesis, regulation of epithelial cell proliferation and differentiation, mucosal protection, wound healing and extracellular matrix (ECM) metabolism that affects the growth of the basement membrane[81,82]. In addition, many inflammatory cells, such as macrophages and lymphocytes, are also important components of the microenvironment in both normal and pathological states. PCMFs and inflammatory cells are located immediately beneath the basement membrane, just under the epithelial cells, and function through the secretion of growth factors, cytokines and basement membrane/ECM proteins; they become activated through direct and indirect interactions[82]. Several studies showed that interstitial myofibroblasts and inflammatory cells increase in neoplastic lesions, suggesting that myofibroblasts and inflammatory cells play critical roles in colorectal neoplasia, as well as under normal conditions[83-85].

The stem/progenitor cells, transient amplifying cells, and, finally, the terminally differentiated cells in intestinal crypts are under homeostatic control through important signals, such as the Wnt, bone morphogenic protein (BMP), NOTCH, Hh and phosphoinositol 3-kinase (PI3K)/mTOR pathways[86]. The regulation of these signals occurs *via* the tight control of interactions between stem/progenitor cells and the niche microenvironment, such as the PCMFs, smooth muscle cells and inflammatory cells[86]. The dysregulation of cryptal homeostasis can induce tumorigenesis and the micro environmental factors secreted by inflammatory cells and myofibroblasts in tumors, as well as accumulation of epithelial changes, have important roles in early tumor progression[86].

Recent evidence suggests that CRC may arise from mutated colorectal stem or progenitor cells that have been termed colorectal cancer stem cells (CSCs) or initiating cells because of their exclusive ability to sustain tumor formation[87,88]. Colorectal CSCs have been identified based on the expression of specific cell surface markers, such as cluster of differentiation (CD)133, CD44, CD166, aldehyde dehydrogenase, doublecortin-like kinase 1 (Dclk1), Lgr5 and Eph receptor B2, and these cells demonstrated stem/progenitor cell properties, in terms of their ability to self-renew, differentiate, and proliferate indefinitely to drive continuous expansion of the malignant cell population[89-93]. These data emphasize the importance of better characterization of CSCs, because the limited numbers of CSCs within the bulk of the tumor may account for their capability to escape conventional therapies, leading to relapse and metastasis. CSCs are now recognized as a specific target for the complete elimination of CRC (Figure 1). In addition, because these CSCs appear in the very early stages of colorectal carcinogenesis, the early changes that occur in normal and cancer stem cells during carcinogenesis might be an effective target for chemoprevention, as well as treatment of advanced CRC.

Effect and mechanisms of chemopreventive drugs on CSCs

Recent evidence suggests interesting similarities in the effects of the above-mentioned major chemopreventive drugs, such as aspirin, NSAIDs, statins, PPARγ agonists, and metformin; these include their CSC-suppressing effect, anti-inflammatory action, and regulation of altered tumor metabolism. In addition, both anti-inflammatory effects and regulation of altered tumor metabolism are also associated with the CSC-suppressing effects of these drugs.

CSCs are involved in tumor initiation, growth, recurrence, and metastasis; therefore, these data suggest that the preventive and survival-improving effects of chemopreventive drugs on CRC might be related to their CSC-suppressing ability. Therefore, in this section, I focus on the relationship between normal/cancer stem cell-related pathways and the mechanism of the chemopreventive drugs.

Direct effect on stem cell and cancer stem cells via regulation of Wnt, NOTCH and BMP pathways

The anticarcinogenic activity of NSAIDs in CRC may depend mostly on the inhibition of COX2 activity, because prostaglandins (PGs) play an important role in tumorigenesis in CRC[94-97]. COX2 is reported to be overexpressed in 85% of human CRC cases and in about 50% of colorectal adenomas[94], and was also identified in an animal model[97], in which a *COX2* null mutation significantly reduced the number and size of polyps in $Apc^{\Delta 716}$ mice[98]. An earlier study reported that COX2 over-expression leads to the production of PGE2, which ultimately stimulates β-catenin-mediated transcription in colon cancer[99]. The WNT/β-catenin pathway is thought to be involved in the regulation of CSCs, and is one of the most interesting therapeutic targets in CSCs[100]. In terms of the anti-CSC effect of NSAIDs, Moon *et al*[101] showed that the anti-CSC effects of NSAIDs were related to both COX2-dependent and -independent pathways.

As traditional NSAIDs exert anticancer effects *via* COX2-independent mechanisms[102], the COX2-independent pathways of NSAIDs could be involved in their anti-CSC activity. In several previous reports, NSAIDs were shown to inhibit NOTCH/hairy and enhancer of split 1 (HES1) signaling pathway as a γ-secretase inhibitor[103,104] and activate the PPARγ expression as a PPARγ agonist[105,106]. NOTCH/HES1 signaling has been shown to be oncogenic in CRCs, inhibiting the terminal differentiation of epithelial cells[107], and the dysregulation of the NOTCH/HES1 signaling was implicated in the self-renewal and maintenance of CSCs in CRC[108]. Meanwhile, PPARγ activation resulted in growth arrest and induced differentiation of colon cancer cells[45]. In addition, the CSC-inhibitory effect of PPARγ agonists through the inhibition of the Janus kinase-signal transducer and activator of transcription (STAT) pathway was demonstrated

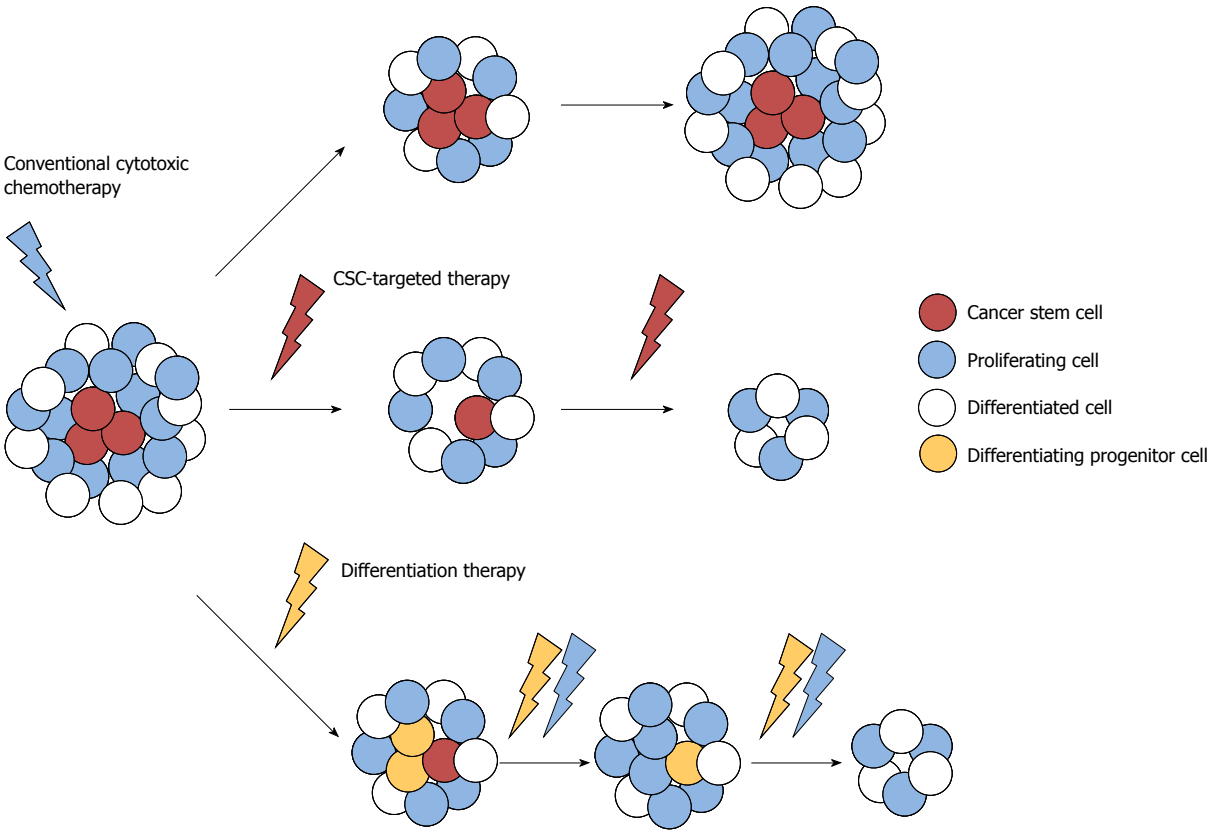

Figure 1 Role of cancer stem cell and anti-cancer stem cell therapy in colorectal cancer. The limited numbers of cancer stem cell (CSC) within the bulk of the tumor may account for their capability to escape conventional therapies, leading to relapse and metastasis; therefore, CSCs are now recognized as a specific target for the complete elimination of colorectal cancer. CSCs are resistant to conventional chemotherapy: the tumor is reduced in size in the short term, but eventually relapses, driven by CSCs. When CSC-targeted therapy or CSC-differentiating therapy is combined with conventional therapy, a tumor will progressively exhaust its growth potential.

in brain CSCs[109]. In this context, Moon *et al*[101] showed that the NOTCH pathway and PPARγ could be related to CSCs in CRC and be down- and up-regulated by NSAIDs, respectively, suggesting that NSAIDs suppress colon CSCs *via* COX2-independent pathways.

In addition, Qiu *et al*[110] demonstrated that sulindac induces apoptosis to remove the intestinal stem cells with aberrant Wnt signaling, and that diablo IAP-binding mitochondrial protein (also referred to as SMAC), a mitochondrial apoptogenic protein, has a central role in this tumor-suppressive effect of sulindac. These results suggested that the chemopreventive effect of NSAIDs is mediated through the elimination of stem cells that are inappropriately activated by oncogenic events.

Some natural chemopreventive dietary compounds, such as curcumin, sulforaphane and piperine, also have been shown to suppress CSCs through inhibition of WNT/β-catenin signaling[111-113].

Statins also have anti-CSC activity. Kodach *et al*[114] showed that tumor-suppressive BMP signaling was silenced by promoter hypermethylation of BMP2 in CRC, and downregulation of DNA methyltransferase activity by statin led to BMP2 promoter demethylation and upregulation of BMP2 expression, culminating in the differentiation of CRC cells and reduction of "stemness".

Therefore, the suppression of Wnt and NOTCH signaling, and activation of BMP and PPARγ signaling that is induced by NSAIDs, statins, PPARγ agonists, and some natural chemopreventive dietary compounds might have potential effects on the fate of stem cells, inducing cell differentiation, cell cycle arrest, and apoptosis.

Regulation of stem cell niche and the inflammatory nuclear factor-κB pathway

Recent studies show that bidirectional conversion between CSCs and non-CSCs can be triggered by stromal factors secreted by inflammatory cells or myofibroblasts in the tumor microenvironment[115,116]. These factors enhance Wnt activation, induce dedifferentiation of non-stem cells and expand stem cell properties during tumorigenesis. In this regard, the anti-inflammatory effect of chemopreventive drugs can retard this de-differentiating effect.

As an important component of the tumor microenvironment, chronic inflammation is also a key factor in the progression of many cancers. Nuclear factor (NF)-κB represents a key transcription factor within the inflammatory tumor microenvironment. Schwitalla *et al*[116] demonstrated NF-κB's function in CSCs, showing that elevated NF-κB signaling enhances Wnt activation and induces de-differentiation of non-stem cells that have acquired a tumor-initiating capacity. Subsequently, epithelial cell-specific ablation of the RelA/p65 subunit of NF-κB retards crypt stem cell expansion; these data support the

concept of bidirectional conversion and the importance of inflammatory signaling for de-differentiation and generation of CSCs[116].

Metformin inhibits initial cellular transformation and selectively suppresses CSCs by inhibition of NF-κB and STAT3[117]. In addition, metformin reduces inflammatory responses *via* inhibition of tumor necrosis factor (TNF)-production in human monocytes[118], and metformin-induced AMPK signaling inhibits the NF-κB-mediated inflammatory responses[119]. Thus, metformin may target the inflammatory processes in the microenvironment of most neoplastic tissues and cancer cells[117,119].

Similarly, PPARγ agonists can attenuate NF-κB-dependent signaling and induce downregulation of pro-inflammatory target genes, such as TNF and interleukin-6[120], and statins also reduce colon tumorigenesis *via* their potential anti-inflammatory and immunomodulatory properties[121,122]. In addition, the anti-inflammatory action of NSAIDs is already well established. All the major chemopreventive drugs discussed in this review have anti-inflammatory properties, which suggest their association with anti-CSC activity through an anti-inflammatory action on the tumor microenvironment, along with their direct effects on CSCs.

Vermeulen *et al*[115] demonstrated that myofibroblast-secreted factors, specifically hepatocyte growth factor, activate β-catenin-dependent transcription and subsequently CSC clonogenicity, indicating that Wnt activity and cancer stemness are regulated by microenvironmental factors. They also showed that myofibroblast-secreted factors restore the CSC phenotype in more differentiated cells, suggesting the dynamic bidirectional conversion of stemness of colon cancer cells[115].

Currently, the regulation of microenvironmetal factors has become one of the major targets for development of anti-CSC drugs, and they could be important targets for chemoprevention as well, because the stem cell niche is involved in the very early stages of tumorigenesis.

Regulation of altered tumor metabolism and the AMPK/mTOR pathway

In terms of cancer metabolism, recent evidence reveals that metabolic alterations and reprogramming of cancer cells are not indirect responses to cell proliferation, but altered metabolism itself can be tumorigenic by changing cell signaling and blocking cellular differentiation[123].

The AMPK/mTOR pathway is a central cellular energy sensor[124]. Liver kinase B1 (LKB1), the upstream activator of AMPK, is a tumor suppressor, and the major pathway controlled by LKB1-AMPK activation is the mTOR signaling pathway, which regulates cell growth and proliferation[124,125]. Activation of AMPK led to inhibition of the mTOR through phosphorylation and subsequent activation of the tumor suppressor tuberous sclerosis complex 2. mTOR is a key regulator of growth factor and nutrient signals, as well as a critical mediator of the PI3K/protein kinase B/Akt pathway, one of the most frequently dysregulated signaling pathways in human cancer[124,126].

In preclinical studies, metformin induced tumor suppression through mTOR inhibition by AMPK activation[127]. In addition, activation of the Akt/mTOR pathway has been associated with malignant progression, resistance to many types of cancer therapy and poor prognosis[126]. The PI3K/Akt/mTOR pathway has, therefore, recently been identified as a target for novel cancer therapy, and the inhibition of mTOR signaling is thought to be one of the major mechanisms involving the anti-cancer effect of metformin. Activation of AMPK also induces cell cycle arrest by inhibiting the expression of cyclin D1 and activating p21/p27, resulting in cellular senescence, quiescence, and apoptosis[128,129].

Recent studies have demonstrated that metformin could selectively suppress cancer stem cells using *in vitro* experiments and *in vivo* xenograft models[117,130,131]. Metformin has also been shown to improve tumor response to chemotherapy, by activating a cytotoxic effect on CSCs that exhibit chemoresistant features[117,130,132]. The PI3K/Akt/mTOR pathway is activated for maintenance and proliferation of CSCs[133-135]; therefore, the mechanism of action of metformin-induced CSC suppression involves the activation of AMPK and the consequent inactivation of mTOR. CSCs are known to be resistant to conventional chemotherapy, and are a cause of cancer recurrence and metastasis; therefore, metformin's effect of eliminating cancer stem cells suggest the possibility of metformin as an adjunctive agent combined with conventional chemotherapy, as well as a chemopreventive drug.

Moreover, aspirin, statin, and PPARγ agonists also induce the activation of AMPK, targeting regulators of intracellular energy homeostasis and metabolism[136-140]. These could contribute to their protective effects against development of CRC.

CONCLUSION

Candidates for effective CRC chemopreventive drugs have been identified through epidemiological studies, basic research and clinical trials. However, to develop more effective chemopreventive drugs with good safety profiles, we need to target cells and pathways that are essential and critical to carcinogenesis, along with targeting the traditional multi-step process of CRC tumorigenesis.

With recent advances in our understanding of intestinal crypt homeostasis and its dysregulation, mutated stem cells and CSCs in early carcinogenesis are likely to be promising targets for chemoprevention of CRC. However, to target the CSCs and stem cell-specific signaling pathways in early carcinogenesis, a detailed understanding of the mechanisms of stem cell maintenance and differentiation, and their relationship with the carcinogenic pathways is needed.

Several recent reports indicate that major chemopreventive drugs like aspirin, NSAIDs, statins, PPARγ agonists, and metformin have CSC-suppressing effects

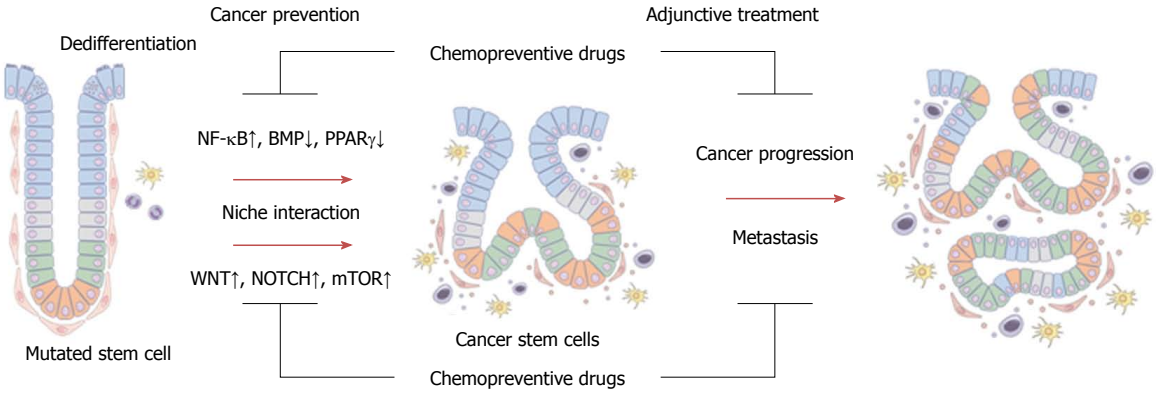

Figure 2 Effects of chemopreventive drugs acting on cancer stem cells and their related signaling pathways in colorectal carcinogenesis. Changes and crosstalk in the stem cells, microenvironment, and metabolism are closely related to essential steps in early carcinogenesis and tumor progression. Mutated stem cells and dedifferentiated stem-like cells can progress to cancer stem cells through dysregulation of stem cell-regulating pathways (Wnt, NOTCH, and BMP), interaction with stem cell niche or tumor microenvironment (inflammatory NF-κB and stromal factor-induced Wnt pathways), and alteration of tumor metabolism [AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway]. The major chemopreventive drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), statins, PPARγ agonists, and metformin, have cancer stem cell (CSC)-suppressing effects via regulation of these pathways. The development of CSC-suppressing chemopreventive drugs could be useful for adjunctive treatment to avoid relapse or metastasis, as well as more potent chemoprevention in colorectal cancer. PPAR: Peroxisome proliferator-activated receptor; NF: Nuclear factor; BMP: Bone morphogenic protein.

via regulation of stem cell-regulating pathways (Wnt, NOTCH, and BMP), stem cell niche or tumor microenvironment (inflammatory NF-κB and stromal factor-induced Wnt pathways) and altered tumor metabolism (AMPK/mTOR pathway) (Figure 2).

Changes in the stem cells, microenvironment, and metabolism are closely related, underlying essential steps in early carcinogenesis and tumor progression, and could be critical targets for chemoprevention and treatment of CRC (Figure 2). In addition to being more effective antineoplastic and chemopreventive drugs, either alone or in combination with other agents, these chemopreventive drugs could also be the basis for development of chemically modified drugs with better chemopreventive activity and a more desirable safety profile.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Current and future molecular diagnostics in colorectal cancer and colorectal adenoma

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Abstract

Colorectal cancer (CRC) is one of the most prevalent cancers in developed countries. On the other hand, CRC is also one of the most curable cancers if it is detected in early stages through regular colonoscopy or sigmoidoscopy. Since CRC develops slowly from precancerous lesions, early detection can reduce both the incidence and mortality of the disease. Fecal occult blood test is a widely used non-invasive screening tool for CRC. Although fecal occult blood test is simple and cost-effective in screening CRC, there is room for improvement in terms of the accuracy of the test. Genetic dysregulations have been found to play an important role in CRC development. With better understanding of the molecular basis of CRC, there is a growing expectation on the development of diagnostic tests based on more sensitive and specific molecular markers and those tests may provide a breakthrough to the limitations of current screening tests for CRC. In this review, the molecular basis of CRC development, the characteristics and applications of different non-invasive molecular biomarkers, as well as the technologies available for the detection were discussed. This review intended to provide a summary on the current and future molecular diagnostics in CRC and its pre-malignant state, colorectal adenoma.

Key words: Colorectal cancer; Colorectal adenoma; Molecular diagnostics; Fecal occult blood test; Non-invasive

Core tip: In this review, the molecular basis of colorectal cancer (CRC) development, the characteristics and applications of different non-invasive molecular biomarkers, as well as the technologies available for the detection were discussed. This review intended to provide a summary on the current and future molecular diagnostics in CRC and its pre-malignant state, colorectal adenoma.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers and cause of cancer mortality in developed countries[1]. Fortunately, CRC is also one of the most curable cancers if it is detected in early stage through regular colonoscopy[2]. Genetic mutations play important roles in CRC development[3]. Recently, different initiating genes have been found to be involved in different categories of CRC and most of the genetic mutations are somatic with no implication for future generations[4]. Nevertheless, studies on monozygotic twins have revealed that about 35% of CRC can be attributed to genetic susceptibility[5]. Based on the above findings, CRC development is most likely caused by genetic-environment interaction.

CRC can be broadly classified into two categories, one related to hereditary while another is non-hereditary (sporadic)[6,7]. Hereditary CRC can further be classified into two sub-groups, *i.e.*, hereditary non-polyposis colorectal cancer (HNPCC) which comprises about one to six percent of all colorectal cancer[8] and multiple polyps CRC, which includes familial adenomatous polyposis (FAP), hamartomatous polyposis syndrome and MUTYH-associated adenomatous polyposis[6]. Each of the above mentioned CRC subtypes involve different genetic causes[6]. Conventionally, fecal occult blood testing (FOBT) was widely used as a non-invasive screening tool for CRC[9]. Although FOBT is simple and inexpensive, it is not an effective tool for screening CRC as false positive results might be yield by diet and medication. Immunological FOBT that detects human haemoglobin, although specific, shows low sensitivity at detecting adenomas and CRC[10]. Invasive screening tests such as colonoscopy are more effective and sensitive in screening CRC, however, high cost and inconvenience limits the diagnostic value since they require extensive bowel preparation and invasion of privacy[10]. Following the better understanding in the molecular basis of CRC, molecular markers testing may be an alternative to FOBT in non-invasive CRC screening.

EPIDEMIOLOGY

Studies showed that the incidence rate of CRC was higher in men and the male-to-female incidence rate ratio has increased progressively[11]. However, the rate of incidence among races has not been frequently reported[12]. The peak incidence for CRC was found to be between ages of 61 and 70[11]. Approximately 6% of the incidence occurred before age of 30 is possibly hereditary CRC rather than sporadic CRC[13]. If CRC is found in young patients, pre-existing polyposis syndrome may be suspected[14]. The death rate of CRC is highest in the United States, Australia, New Zealand and Eastern European countries while comparatively lower in Mexico, South America and Africa[14]. Such a difference in the rate of incidence has been suggested to be related to their lifestyles. Studies also showed that dietary practices, obesity and physical inactivity are the risk factors for CRC[15,16].

According to the Hong Kong Cancer Registry, the incidence of CRC in Hong Kong was 4335 (16.6% of all cancers) in 2012 and it was the second most common cancer in Hong Kong[17]. There is a rising concern on the importance of the early diagnosis of CRC.

CLINICAL FEATURES

Many CRCs remain asymptomatic for years before diagnosis. However, as for cecal and right colonic cancers that cause fatigue, weakness and iron-deficiency anemia, early detection of the disease may be possible at early stage and these bulky lesions bleed readily. Concerning left-sided lesions, occult bleeding, changes in bowel habit, left quadrant discomfort and weight loss may develop. Furthermore, there is a higher chance for early discovery and hence successful removal of the left-sided lesions since patients have prominent disturbance in bowel function such as diarrhea and constipation. However, the localization of the bowel segments and the more infiltrative nature of rectum and sigmoid will reduce the chance of CRC detection. Colorectal tumors spread to other parts of the body by direct extension into adjacent structures and metastasis through the lymphatics and blood vessels. According to the previous studies, the favored metastatic sites of CRC are lymph nodes, liver, lung and bones[14]. Currently, the most commonly used system to describe the extent of CRC is the tumor-nodes-metastasis (TNM) classification and staging system released by the American Joint Commission on Cancer[18].

MOLECULAR BASIS OF COLORECTAL CANCER

CRC is believed to be caused by a cascade of genetic mutations. The typical model for the carcinogenesis of CRC proposed by Fearon *et al*[19] can be described as adenoma-carcinoma sequence[6]. Adenoma-carcinoma sequence describes a gradual progression from normal epithelial mucosa to adenoma and then to carcinoma as a result of a series of genetic changes such as mutation and gene amplification[20] (Figure 1). The risk of recurrence and subsequent death due to CRC is closely related to the stage of the disease at the time of the first diagnosis[6]. Recent studies showed the risk of death from CRC could be reduced by shifting the detection of the disease to an earlier stage *via* mass screening and intervening[20]. Therefore, there is an urgent need for biomarkers for early detection of CRC[21].

In the molecular aspects, CRC is caused by the loss of genomic stability that drives the development of CRC by facilitating the acquirement of tumor-associated mu-

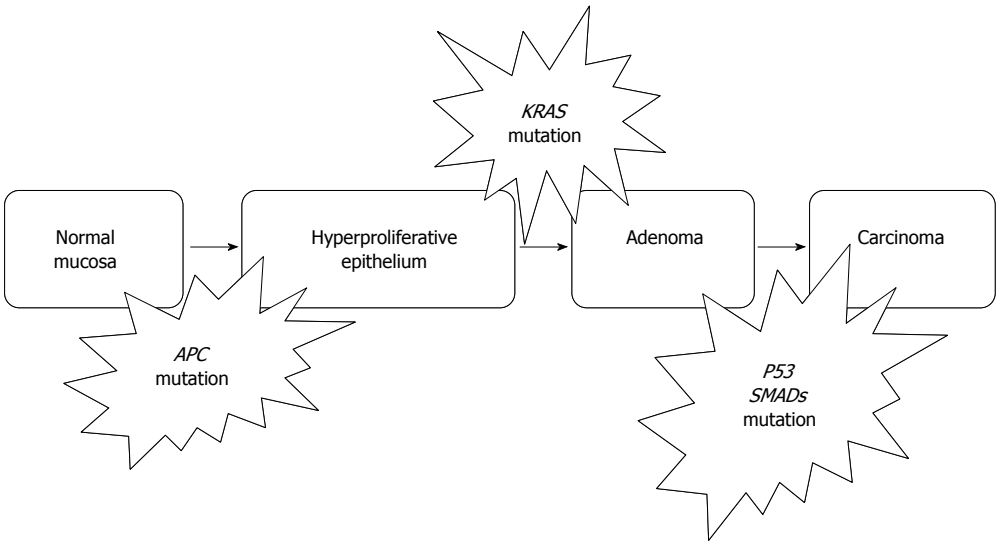

Figure 1 Adenoma-carcinoma sequence in colorectal cancer formation. This is a simplified presentation of colorectal cancer tumourigenesis. The true carcinogenesis progress of colorectal cancer is much more complicated.

tations. In CRC or adenoma, several forms of genomic instability have been identified, including chromosomal instability (CIN), microsatellite instability (MSI), and epigenetic gene silencing.

CIN

Basically, CIN is characterized by any chromosomal copy number or structure change. CIN is the commonest genomic instability that encompasses 80% to 85% of all CRC and adenoma[6]. These types of genetic problem always result in abnormal karyotypes such as aneuploidy, chromosome rearrangement, oncogene activation and loss of heterozygosity of tumor suppressor genes[6]. It is suggested that CIN induces carcinoma through the loss or mutation of tumor suppressor genes such as *APC*, *TP53* and also through activation of oncogenes such as *KRAS*[22]. CRC caused by CIN usually have poor prognosis[23].

MSI

MSI accounts for 15% of CRC[6]. It is characterized by altered length of gene with small deletions and insertions of short repetitive deoxyribonucleic acid (DNA) sequences (microsatellite) distributed throughout the genome[6,24]. Single MSI within the whole genome may have no significant effects but accumulation of the mutations can result in frame shifts within gene coding sequences and the subsequent inactivation of the genes would give rise to the progression of tumor[6]. Mutations are frequently found in the coding mononucleotide repeats of tumor suppressors such as transforming growth factor βR2 (69%) and activin receptor type 2 (83%). Unlike the tumor caused by chromosomal instability, these kinds of tumors are diploid or near-diploid[6].

The underlying cause of MSI can be explained by two mechanisms[6]. The first mechanism is the defective mismatch repair (MMR) system, in which both alleles of a *MMR* gene (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) are nonfunctional. This results in the loss of ability to repair DNA replication mismatches in the affected cells[22]. The germline mutation of the *MMR* gene can lead to HNPCC[6]. The second mechanism is the hypermethylation of promoter in *MMR* genes that suppress the expression of the genes like *MLH1*[22].

Epigenetic gene silencing

Epigenetic silencing of genes is mostly caused by DNA methylation[6]. Cancers with high degrees of methylation can be considered as CpG island methylation phenotype (CIMP) positive[25], and CIMP encompasses 35%-40% of sporadic CRC[6].

DNA methylation is involved in normal cellular control of gene expression[6]. A vast majority of methylated cytosines in the human genome are found in the CpG dinucleotide sequences. In normal cells, dense regions of CpG sequences (CpG island) are usually found in the regions close to promoters[6]. The methylation patterns of these CpG sequences are gene-specific. Aberrant CpG hypermethylation can lead to silencing of tumor-suppressor genes in carcinogenesis since the expression of the genes is repressed[25]. For example, *p16*, *p14*, *MGMT* and *hMLH1* are commonly silenced genes in CRC patients[26]. In some cases, the presence of epigenetic silencing is overlapped with MSI[22] (Figure 2). Some sporadic CRC with microsatellite instability is caused by DNA methylation. For example, DNA methylation of *MLH1* gene promoter blocks its expression and destroys the ability of MMR system[27].

Hereditary CRC

HNPCC: HNPCC is the most common hereditary colon cancer syndrome. It is autosomal dominant[28]. The presence of HNPCC is defined as the presence of germ-line mutation found in one of the four *MMR* genes, namely *MLH1*, *MSH2*, *MSH6* and *PMS2*[29]. Bethesda guidelines can be used as a screening tool for HNPCC[30].

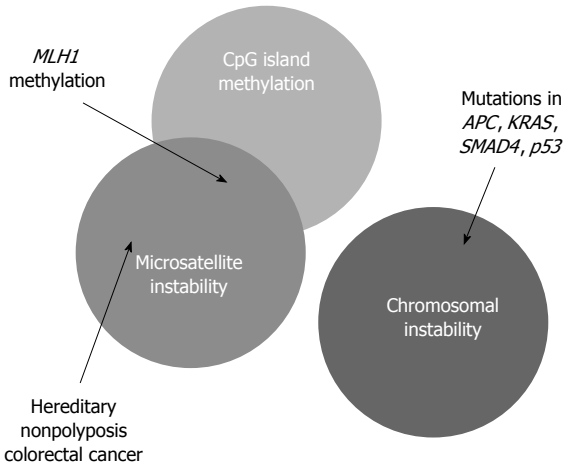

Figure 2 Genetic instability pathways that drive colorectal cancer development.

FAP: FAP is the most common polyposis syndrome. It is autosomal dominant and is caused by *de novo* germline mutations[31]. The presence of FAP can be diagnosed by direct sequencing of the germ-line mutations in *APC* gene on chromosome 5q21[32].

CLINICAL APPLICATION OF MOLECULAR MARKERS

CRC develops slowly *via* accumulation of genetic mutations. Therefore, many CRCs remain asymptomatic for years before diagnosis. Carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) are the two most investigated gastrointestinal tumor markers of CRC. Although the high levels of the CEA and CA are associated with cancer progression in CRC, they may not be detected until the cancer is in advanced stages[10]. Moreover, the levels of those markers may also elevate in response to other diseases[10]. For example, high level of CEA may also be found in patients with inflammatory bowel disease. To conclude, CEA and CA are not effective in early detection of CRC. They should be used as prognostic markers rather than diagnostic markers.

Detection of CRC in early stages can reduce both the incidence and mortality of the disease. Molecular markers that detect gene mutation in the early stages of CRC can be used as non-invasive screening tests for early detection of CRC, followed by invasive confirmatory tests such as colonoscopy for individuals with positive results.

CURRENT MOLECULAR DIAGNOSTICS IN COLORECTAL CANCER AND COLORECTAL ADENOMA

Current markers

Since hereditary and sporadic forms of CRC are clinically different, the following discussions are focused on sporadic CRC.

CIN tumor marker: RAS family is a series of genes including *HRAS*, *NRAS* and *KRAS*[22]. The RAS family of oncogenes encode plasma membrane proteins at inner surface[6]. In RAS family, *KRAS* gene plays the most important role. *KRAS* gene encodes GTP (guanosine 5'-triphosphate)-binding proteins, which function as molecular switches and act as self-inactivating intracellular signal transducers for surface receptors such as epidermal growth factor receptor. The activation of *RAS* genes can promote cell survival and suppress apoptosis[22].

The oncogenic mutations of *RAS* family are believed to be an early event in CRC and they occur in 37% of CRCs and approximately 50% of adenomas > 1 cm (9% in adenomas < 1 cm)[23]. Most of *KRAS* mutations occur in codon 12 (70%-80%) and codon 13[23,33]. In clinical applications, *KRAS* mutation analysis is widely used as a prognostic and predictive biomarker of anti-EGFR monoclonal antibodies like cetuximab and panitumumab to predict the therapeutic effectiveness in CRC[34].

As well as *KRAS*, recent clinical studies start to focus on v-raf murine sarcoma viral oncogene homologue B1 (*BRAF*) and neuroblastoma-ras (*NRAS*). *BRAF* genes encode serine/threonine protein kinase that is regulated by KRA protein[35]. Mutation in *BRAF* gene occurs in approximately 12% of all CRC patients and it is mutually exclusive of *KRAS* mutation[35]. Recent studies try to investigate the clinical importance of *BRAF* mutation as a prognostic and predictive marker to predict resistance to anti-EGFR therapy[36]. Consideration of *BRAF* mutation is also recommended when *KRAS* mutation is not found[37]. *NRAS* is closely related to *KRAS*. *NRAS* mutation occurs in codon 61 and it is found in approximately 3%-5% of all CRC patients[38]. Similar to *BRAF* mutation, *NRAS* mutation is mutually exclusive of *KRAS* mutation[37,38].

Adenomatous polyposis coli (*APC*) gene plays a crucial role in the Wnt/Wingless pathway. *APC* gene is the most important gatekeeper of colonic epithelial cell proliferation and it is responsible for controlling the underlying oncoprotein called β-catenin. The loss of function in *APC* gene may lead to the transition of adenoma from normal colonic mucosa due to the up-regulation of β-catenin[22].

Germline *APC* mutations usually give rise to FAP. It is an inherited cancer-associated disorder in which more than 100 adenomatous polyps can be developed in mutant gene carriers. For patient with FAP, the risk of CRC by the age of 40 years is almost 100%. Somatic *APC* mutations are present in most sporadic colorectal adenomas and cancers. Similar to *KRAS*, *APC* mutations appear in the early stage of the progression from adenoma to carcinoma. Therefore, mutations in the *APC* gene are good biomarkers for identifying individuals at risk of CRC in patients' families so as to guide the frequency of CRC surveillance and the recommendation of prophylactic surgery[22].

MSI markers: Both polymerase chain reaction (PCR)

Table 1 Panel with five microsatellite markers

Microsatellite	Forward primer	Reverse primer
BAT25	5′-TCGCCTCCAAGAATGTAAGT-3′	5′-TCTGCATTTTAACTATGGCTC-3′
BAT26	5′-TGACTACTTTTGACTTCAGCC-3′	5′-AACCATTCAACATTTTTAACCC-3′
D2S123	5′-AAACAGGATGCCTGCCTTTA-3′	5′-GGACTTTCCACCTATGGGAC-3′
D5S346	5′-AGCAGATAAGACAGTATTACTAGTT-3′	5′-ACTCACTCTAGTGATAAATCGGG-3′
D17S250	5′-GGAAGAATCAAATAGACAAT-3′	5′-GCTGGCCATATATATATTTAAACC-3′

based methods and immunounhistochemistry can be used to detect MSI. Immunounhistochemistry staining detects DNA MMR system proteins such as MLH1 and MSH2. The loss in these markers is indicative of MSI. However, immunounhistochemistry staining is only best performed when the resection specimens are fixed promptly and properly since the quality of staining would be affected. Furthermore, the size of the specimens is also a concern in staining. Small specimens are not suitable for staining since the amount of tumor cells and internal staining controls are limited[6].

As PCR-based methods, currently, a 5 biomarkers panel MSI test, so-called Bethesda markers, has been developed to assess MSI status (Table 1). It consists of 2 mononucleotide loci (*BAT25* and *BAT26*) and 3 dinucleotide loci (*D2S123*, *D5S346*, and *D17S250*)[39]. This panel is useful to detect about 15% of all CRC due to germline mutation in one of the mismatch repair genes (*MLH1, MSH2, MSH6* and *PMS2*) or epigenetic slicing of *MLH1*. Among the five tested regions, when MSIs are present in two or more regions, the tumor is classified as MSI-High. Otherwise, the tumor is considered as MSI -Low (MSI in one region) or MSI-Stable (no MSI)[24].

The above classification system is highly valuable in prognosis and therapy since standard chemotherapy using 5-fluorouracil is not effective in treating MSI-High tumor. Instead, irinotecan-containing regimens have improved response and better prognosis for MSI-High tumor[23].

Epigenetic markers: CpG regions that are hypermethylated in CRC patients when compared to normal individuals are valuable for biomarker development. Methylations on different regions of DNA promoter have been shown to be involved in the early event of CRC development. *APC* methylation is one of the examples. In addition, the methylation of *MLH1* associated silencing is widely used as prognostic and predictive markers for CRC[6].

Septin 9 (*SEPT9*) gene encodes the septin 9 protein, which is a member of GTP-binding proteins that involves in many cellular processes such as cell cycle. Disruption of *SEPT9* gene could result in tumor formation[40]. In CRC development, the promoter region of *SEPT9* gene was found to be hypermethylated at the early stage of CRC[41]. In the blood samples of CRC patients, the level of circulating methylated *SEPT9* DNA sequences was found to be increased when compared to those of normal controls. This elevation was possibly due to apoptotic release of DNA from tumor cells into the bloodstream. The methylated *SEPT9* DNA has been reported to be an effective diagnostic marker for the non-invasive detection of CRC that is independent to the ages and genders of the patients[41-44].

Unfragmented long-form DNA: In normal colonocytes, DNA is digested during apoptosis and some DNA fragments of 180-200 bps long is released into stool. However, cancerous cells in CRC show a decreased rate of apoptosis and they are not affected by the normal action of nuclear endonuclease[9]. As a result, intact unfragmented DNA sequences with 1800-2400 bp are detected in stool samples when colonocytes are shed into the lumen and then fecal stream[9,45]. The detection of unfragmented long DNA molecules in stool samples could therefore be used for CRC screening in additional to FOBT[9].

Current molecular diagnostics methods

Methods for detection of gene mutation: There are a number of molecular techniques available to detect gene mutation in CRC such as allele-specific PCR, also known as amplification refractory mutation system (ARMS) that detects specific known mutated form of a gene and real-time quantitative PCR.

Both Sanger sequencing and allele-specific PCR can be used to detect the gene mutations mentioned above, such as *APC* and *KRAS* mutations. Sanger sequencing directly detects nucleotide sequences of regions of interest. Both known and novel nucleotide changes, including base substitution, insertion and deletion mutations, could be detected by this method. However, the long turnover time and high running cost make it not suitable for routine clinical uses. Allele-specific PCR is another choice for the detection of gene mutations with known positions and base changes (Figure 3). The method makes use of the allele-specific primers to selectively amplify the mutational allele sequences. Allele-specific PCR could be adopted to a real-time PCR platform in order to increase the speed and accuracy of the detection[46]. Real-time PCR employs a fluorogenic probe that is specific to the targeted DNA sequence. The probe consists of a fluorophore and a quencher attached covalently on both ends. When the probe is intact, the quencher masks any fluorescent signal emitted by the fluorophore. During PCR, the probe is first annealed to the target sequence, and then broken down by the 5' to 3' exonuclease activity of DNA polymerase. After the degradation of the probe, the fluorophore is released from the quencher and fluorescence is detected in real time (Figure 4). By detecting the change in fluores-

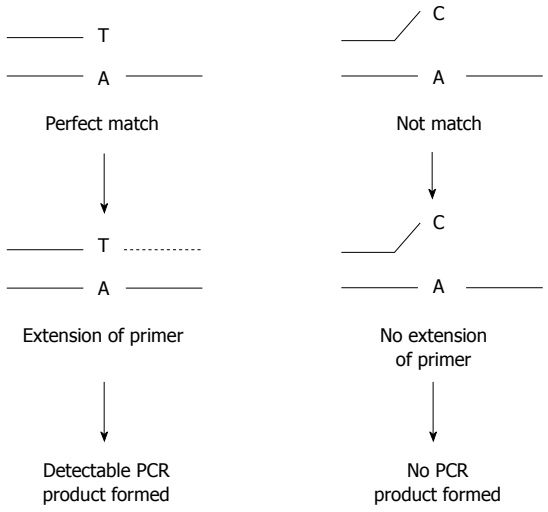

Figure 3 Principle of allele-specific polymerase chain reaction. PCR: Polymerase chain reaction.

cent signal, the amount of target DNA molecules can be measured. With the help of real-time PCR, screening of genes mutations can be performed in batch mode and the turnover time is shorter than that of Sanger sequencing[6]. Compared to conventional PCR, real-time PCR is quantitative, fast and sensitive. As no post-PCR manipulation, such as gel or capillary electrophoresis, is required, the risk of laboratory cross-contamination can be minimized. However, real-time PCR is not suitable for studying microsatellite in which the analysis of amplification product size is required.

Microsatellite markers detection: PCR amplification of specific microsatellite repeats is used for MSI detection. The presence of MSI is determined by comparing the length of nucleotide repeats in tumor cells with that of normal cells from the same patient. Normal cells adjacent to the suspicious tumor cells should be collected for comparison. The region of interest is PCR amplified with fluorescent primers and the amplification product is detected by capillary electrophoresis[24].

DNA hypermethylation detection: DNA hypermethylation can be detected in primary colorectal carcinomas using bisulfite conversion of DNA samples followed by methylation-specific PCR. Both quantitative methylation-specific PCR and pyrosequencing techniques can be employed[41,43,47,48].

FUTURE MOLECULAR DIAGNOSTICS IN COLORECTAL CANCER AND COLORECTAL ADENOMA

Choice of biomarkers is very important. In order to develop a new diagnostic method, suitable biomarkers, which are biological substances that can be used to indicate the biological state of a patient, must be identified[10]. A good marker helps the detection of disease at earlier stage so that diseases can be cured effectively. Regarding CRC detection, since CRC is believed to be developed slowly *via* accumulation of genetic mutations, detection of the disease at earlier stage is the key concern for developing new diagnostic methods.

The road to the development of novel biomarkers begins with the discovery of potential candidates. The number of potential candidates produced in the initial stage of development is usually large[44]. Therefore, well designed selection processes are critical to identify clinically significant biomarkers. In selection of novel biomarkers, in terms of CRC detection, a good marker must be capable of discriminating between CRC patients and healthy individuals significantly[44]. As a high-performing screening assay, it must be capable of detecting target analytes at extremely low level. In other words, the clinical sensitivity of the screening assay must be high enough to detect early stage CRC with adequately specificity to the disease[44]. Recommendations for biomarkers studies are given to the investigators by The National Cancer Institute Investigational Drug Steering Committee and United States Food and Drug Administration[49].

MicroRNA markers

MicroRNAs (miRNAs) are small non-coding RNA that is usually 19-23 nucleotides in length. Due to their small sizes, miRNAs are more stable in blood and FFPE tissues than other nucleic acids such as DNA and RNA[50]. miRNAs are involved in post-transcriptional regulation of gene expression. Therefore, they are able to function as oncogenes or tumor suppressor genes, and dysregulation of miRNA would be associated to cancers. Recent studies showed that miRNA circulate in a stable and cell-free form in bloodstream[51]. Therefore, miRNAs that are specific to CRC in blood samples may be identified for the development of non-invasive prognostic and predictive markers of the diseases[52].

MiR-135a and miR-135b play important roles in the regulation of Wnt/Wingless pathway by down-regulating *APC* gene expression[53]. miR-17-3p and miR-92a have been found to be elevated in plasma and CRC tissues in CRC patients, and their levels decreased after removal of the cancer tissues[54]. In the plasma of CRC patients, circulating miR-92 and miR-17 concentrations have been reported to be elevated in the preoperative samples and the concentrations were markedly reduced in the postoperative samples[51]. Those results suggested that circulating miR-92 and miR-17 are potential non-invasive diagnostic markers for CRC. Apart from the miRNAs mentioned above, miR-211 is also believed to be a potential marker for the diagnosis and prognosis of CRC[55].

Methods of miRNA detection

Currently, a number of techniques are available for the detection of miRNA expression. PCR based methods[56], microarrays and *in situ* hybridization are well developed platforms for miRNA profiling. Each technique has its

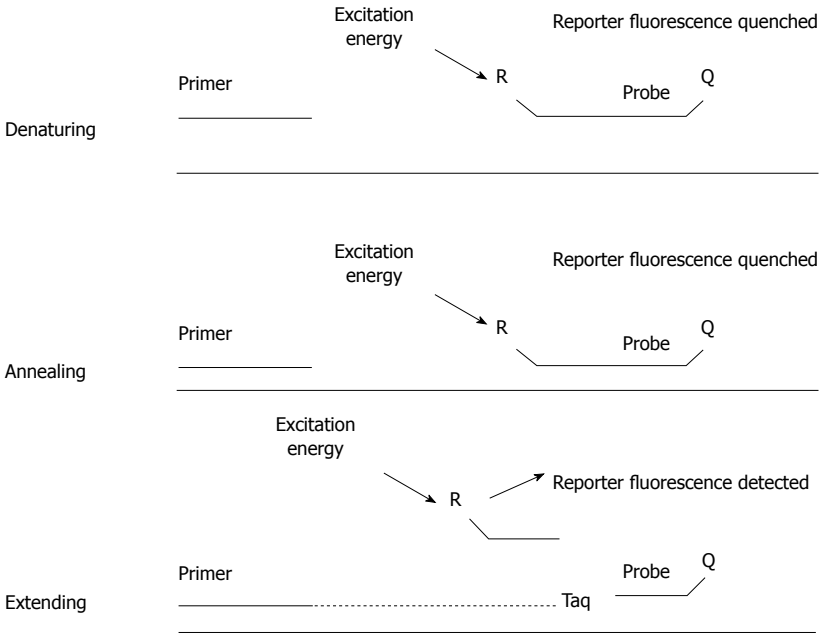

Figure 4 Principle of Taqman real time polymerase chain reaction. R: Reporter; Q: Quencher.

own advantages and disadvantages. Therefore, a suitable technique that suits the requirements of the investigation and experimental conditions should be chosen.

Quantitative reverse transcription PCR: The amount of miRNA in the specimen can be measured by real-time PCR. The first step of the measurement is to reverse transcribe miRNA into cDNA. A stem-loop primer, which enhances the miRNA reverse transcription efficiency by promoting the thermal stability of primer-miRNA complex, is usually employed. After reverse transcription, cDNA molecules are quantified by conventional real-time PCR[57] (Figure 5).

Microarray: MiRNA microarray is a technique based on the hybridization between target miRNAs and an array of predesigned detection probes that are covalently immobilized onto a glass slide. The isolated miRNAs are labeled with fluorescent dye and then hybridized to a miRNA microarray. Fluorescent signal that is emitted from the labeled miRNAs at different positions on the microarray is then detected. By evaluating and analyzing the fluorescence signal data, the identities and relative quantities of miRNAs can be determined[58].

Lateral flow nucleic acid assay: Molecular diagnostic techniques developed for miRNA detection can provide highly specific and sensitive diagnostic results. However, the current existing techniques are too expensive and resource-intensive for the clinics with poor settings to perform the test[59]. In addition, well trained personnel are also a must to carry out the test and analyze the results[60]. In order to put CRC related miRNA screening into routine health check up procedure, a simple and easy-to-use detection method is desired. The emergence of the lateral flow nucleic acid assay and gold nanoparticles (Au-NPs) may help in miRNA detection[60,61]. Lateral flow nucleic acid strip can give a simple, inexpensive, fast and sensitive assay which meets the needs of point-of-care in miRNA detection[59-61].

The principle of the assay is very straightforward. First of all, the specimen is mixed with gold nanoparticles conjugated detection probe (detection probe) and biotin-bridge probe (capture probe). If target miRNA exists in the specimen, after hybridization, both the detection probe and the target miRNA bind to the complementary capture probe. The miRNA-oligonucleotide complex flows down a test strip by capillary action. The complex is eventually captured at the detection zone containing anti-avidin antibody. Nuclease is used to degrade the non-binding capture probe and detection probe[59]. Accumulation of gold nanoparticles causes the development of a red band which can be observed by naked eyes in the detection zone. As the strength of the signal generated is proportional to the amount of the target miRNA within the specimen, to assess the result quantitatively, the strips can be scanned and imaged by a quantitative detection platform[59-61]. In order to increase the sensitivity of the detection, silver enhancement can be used. Researchers have shown that the lateral flow nucleic acid test strips and gold nanoparticles were able to detect and quantify miRNA level in a specimen as low as 1 fmol and 5 amol without and with silver enhancement, respectively[59]. This technique provides a simple, convenient and fast detection for point-of-care detection[60,61].

Protein markers

Novel proteomic technologies have shown promise in identification of new protein markers and profiles for early detection of CRC[62-65]. Changes in protein patterns, either due to the secretion by the tumor tissues or the non-tumor cells in tumor microenvironment, in

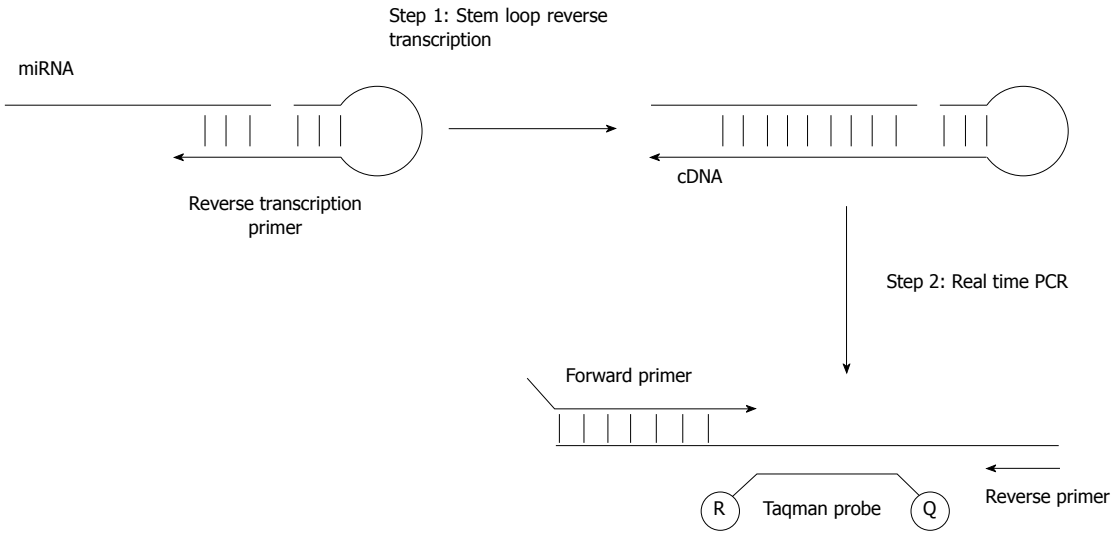

Figure 5 Principle of stem-loop primers. R: Reporter; Q: Quencher; PCR: Polymerase chain reaction.

Table 2 Molecular markers for the detection of colorectal cancer

Markers	Detection method	Specimen	Sensitivity		Specificity	Ref.
KRAS	Mutation analysis	Stool	C	55%	100%	Puig *et al*[67], 2000
			A	27%	100%	
APC gene	Mutation analysis	Stool	C	61%	100%	Traverso *et al*[68], 2002
			A	50%	100%	
MSI markers	Digital-PCR based method	Stool	C	37%	100%	Traverso *et al*[69], 2002
			A	0%		
Long form DNA	Quantitative fluorescence determination by PCR	Stool	C	79%	89%	Calistri *et al*[70], 2009
Septin 9 methylated DNA	Real time PCR analysis	Plasma	C	72%	90%	Grützmann *et al*[42], 2008

C: Colorectal carcinoma; A: Colorectal adenoma; MSI: Microsatellite instability; PCR: Polymerase chain reaction.

the bloodstream can serve as potential cancer markers for CRC detection. With the fast development of proteomics, CRC specific autoantibody patterns and proteomic expression profiles can now be identified by techniques such as matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and surface-enhanced laser desorption/ionization time-of-flight[45]. Proteomic profiling of serum from CRC patients is a promising approach to discriminate CRC patients from healthy individuals[62].

In a previous study, blood samples from individuals diagnosed with CRC and normal healthy individuals were collected. Protein expression spectra were acquired using SELDI mass spectrometry. From the results, increased levels of transferrin, alpha-1-antitrypsin and complement C3a des-arg were identified and they are treated as potential biomarkers for CRC[65]. Similar works were also carried out using either SELDI[66] or MALDI-TOF[62-64].

CONCLUSION

Cancer biomarkers and characteristics of an ideal biomarker for CRC are discussed in this review, as well as the technologies available for their detection. This review aims to summarize the issues on the use of biomarkers for determination of prognosis as well as monitoring of response to therapy. Different molecular biomarkers including markers in stool and blood are discussed[21] (Table 2).

The screening has been shown to be effective in terms of reduction of disease-related mortality and costs. Currently, FOBT is the only screening modality for CRC. DNA-based fecal markers are promising but are not widely used in clinical settings. Detecting abnormal DNA from the tumor cells, which are shed into the fecal stream, can give informative indication on the incidence of CRC. However, complicated environment in stool sample and the presence of PCR inhibitors such as bilirubin and bile acids limits the successful amplification of mutated DNA to a detectable quality[9].

In addition, insufficient sensitivity and specificity preclude the use of all existing serum markers such as carcinoembryonic antigen (CEA) for the early detection of CRC[10]. In the field of clinical research, oncology is expected to have the largest gains from biomarkers over the next five to ten years. Development of personalized medicine for cancer is closely linked to biomarkers, which may serve as the basis for diagnosis, drug discovery and monitoring of diseases. Early detection of CRC not only can help the patients but the healthcare system since expensive chemotherapies can be avoided.

A major challenge in the future development of can-

cer biomarkers will be the integration of proteomics with genomics and metabolomics data and their functional interpretation in conjunction with clinical data and epidemiology[21].

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Predictive and prognostic biomarkers with therapeutic targets in advanced colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the most common human malignant diseases and the second leading cause of cancer-related deaths worldwide. The treatment of advanced CRC has improved significantly in recent years. With the emergence of two targeted antibodies, cetuximab (Erbitux), an anti-epidermal growth factor receptor monoclonal antibody and bevacizumab (Avastin), a vascular endothelial growth factor monoclonal antibody, the treatment of metastatic CRC has entered the era of personalized therapy. Predictive and prognostic biomarkers have, and will continue to, facilitate the selection of suitable patients and the personalization of treatment for metastatic CRC (mCRC). In this review, we will focus primarily on the important progresses made in the personalized treatment of mCRC and discuss the potentially novel predictive and prognostic biomarkers for improved selection of patients for anti-cancer treatment in the future.

Key words: Biomarker; Target therapy; Predictive; Prognostic; Advanced colorectal cancer

Core tip: This review focuses primarily on the important progresses achieved in the personalized treatment of metastatic colorectal cancer and highlights the potentially novel predictive and prognostic biomarkers for improved selection of patients for anti-cancer treatment in the future.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common human malignancies and the second leading cause of cancer-related deaths worldwide. There are 25159 new cases of CRC diagnosed each year and 12161 CRC-related deaths in China[1]. Metastasis to the liver and lung are the main cause of death, with approximately 40%-50% of all patients experiencing metastasis[2,3]. The treatment of advanced CRC has improved significantly in recent years, and the overall survival (OS) for metastatic CRC (mCRC) patients has increased from a median of 10 mo to more than 20 mo[4]. With the emergence of two targeted an-

tibodies, cetuximab (Erbitux), an anti-epidermal growth factor receptor (EGFR) monoclonal antibody and bevacizumab (Avastin), a vascular endothelial growth factor (VEGF) monoclonal antibody, the treatment of mCRC has entered the era of personalized therapy. Treatment on a "personalized" basis now involves a simultaneous case-specific analysis of clinical and pathological characteristics and analysis of a patient's genetic and tumor biomarker profile. Predictive and prognostic biomarkers have, and will continue to, facilitate the selection of suitable patients and the personalization of treatment for mCRC.

A prognostic factor is defined as any parameter, evaluated at diagnosis (or surgery), which is associated with treatment outcome (disease-free interval, survival, local control) and may predict patient outcome independent of treatment. Prognostic factors (biological or clinical) may be defined at any disease stage or setting (for example, performance status in the advanced disease setting). A predictive factor is any parameter which identifies patients who will benefit from a particular treatment and evaluates the response or lack of response to specific treatment. Over the last 30 years, there has been significant advancement in understanding the molecular origins of CRC and the characteristics of tumor aggressiveness[5]. However, in practice, the distinction between prognostic and predictive factors is not straightforward, and many factors are a mixture of the two. Understanding the molecular mechanisms underlying the metastatic process will help us to identify those at the highest risk of recurrence and to find new tumor targets to prevent disease progression.

This review focuses primarily on the important progresses made in the personalized treatment of mCRC and highlights the potentially novel predictive and prognostic biomarkers for improved selection of patients for the anti-cancer treatment in the future.

EGFR

The appropriate use of targeted biologic agents can positively impact a patient's prognosis. Extensive research has focused on tumor factors due to the central role they play in the response to targeted biologic agents. Currently, numerous potential biomarkers are under investigation, and these biomarkers may be clinically useful in the future once validated by appropriate trials (Table 1).

An important molecular target for mCRC treatment is the epidermal growth factor receptor (EGFR). EGFR is a receptor tyrosine kinase frequently expressed in epithelial tumors. Binding of a ligand to the extracellular domain of EGFR activates intracellular signalling *via* several pathways, including the RAS/RAF/MAPK pathway and the PI3K/Akt axis[6]. EGFR is expressed on normal human cells, but higher levels of expression have also been correlated with malignancy in a variety of cancers, including CRC[7]. EGFR has been implicated in colorectal tumorigenesis, tumor progression, and metastasis[8,9]. EGFR is overexpressed in 30%-85% of patients with CRC and has been associated with advanced stage disease. Numerous studies have evaluated the prognostic relevance of EGFR in CRC, but the impact of its expression on survival remains controversial[10]. Two monoclonal antibodies, cetuximab (Erbitux™; Bristol Myers Squibb, Inc., Princeton, NJ, United States) and panitumumab (Vectibix™; Amgen, Inc., Thousand Oaks, CA, United States), target the human EGFR in the treatment of EGFR-overexpressing CRC[11,12]. Genetic alterations of EGFR and its downstream signaling effectors may predict response to anti-EGFR monoclonal antibodies (mAbs), therefore research efforts have been made to understand the specific resistance mechanisms.

The main research areas in this setting have focused on the role of (1) EGFR protein expression; (2) *EGFR* gene copy number; (3) *EGFR* gene mutations; (4) overexpression of *EGFR* ligands (such as epiregulin and amphiregulin); and (5) markers of EGFR downstream signaling[13-17].

Overexpression of EGFR protein, as determined by immunohistochemistry (IHC), was initially selected as an entry criterion for early studies evaluating EGFR inhibitors on the assumption that sensitivity to such agents was associated with EGFR expression[18]. However, a large body of evidence from mCRC patients who were treated with anti-EGFR mAbs[19-21] indicates that this biomarker is poorly associated with response. Moreover, several authors reported that cetuximab was also active in EGFR-negative tumors detected by IHC[22,23]. EGFR expression at either the protein or mRNA level is not correlated with anti-EGFR mAbs response.

In a small fraction of CRCs, *EGFR* overexpression is frequently associated with amplification of the gene (17% in primary and 23% in metastatic tumors)[24]. Activating mutations in the EGFR catalytic domain are seen frequently in lung cancer and play an important role in determining responsiveness to anti-EGFR therapy[25]. However, *EGFR* mutations are very rare in CRC and are not significantly associated with response to anti-EGFR mAbs treatment[26,27].

In contrast, increased *EGFR* gene copy number (EGFR GCN) has been associated with response to anti-EGFR therapy and with prognosis of mCRC in small retrospective studies[28,29]. Recently, Yang *et al*[30] performed a meta-analysis to summarize the evidence for the predictive value of EGFR GCNfor clinical outcomes of mCRC patients treated with anti-EGFR mAbs. The data showed that increased EGFR GCNwas generally associated with a better objective response, especially among patients with wild-type KRAS. In another meta-analysis performed by Jiang *et al*[31], increased EGFR GCN was significantly associated with improved OS and progression-free survival (PFS) in the population that received second-line or higher therapy. The prognostic impact of EGFR GCN on survival does not appear to be related to KRAS status, which suggests that EGFR GCN might be an independent prognostic biomarker. EGFR GCN can

Table 1 Predictive and prognostic biomarkers for biological therapy in metastatic colorectal cancer

Biomarker	Prevalence	Evidence available	Predictive and prognostic value
KRAS mutations	40%	Conclusive	Negative predictive biomarker for anti-EGFR mAbs
		Insufficient	Predicts poor prognosis, but not an independent prognostic factor
BRAF mutations	10%	Substantial	Prognostic marker for poor outcome
		Insufficient	Potential predictive marker for resistance to anti-EGFR mAbs
NRAS mutations	3%-5%	Insufficient[1]	Potential predictive marker for resistance to anti-EGFR mAbs
PIK3CA mutations	15%-20%	Insufficient[1]	Potential predictive marker for resistance to cetuximab (exon 20, not exon 9 mutations)
			Potential prognostic marker for poor outcome
PTEN	20%-40%	Insufficient[1]	Potential predictive marker for resistance to cetuximab
(loss of expression)			Associated with activation of the PIK3CA pathway and adverse disease outcome
P53 mutations	1%-5%	Insufficient[1]	An independent predictive factor for cetuximab benefit
			Not prognostic
Epiregulin, amphiregulin	50%-60%	Insufficient[1]	Associated with resistance to anti-EGFR antibody therapy and adverse clinical outcome
(high expression)			
VEGF-D	40%-75%	Insufficient[1]	Potential predictive marker for response to bevacizumab
VEGF-A		Insufficient[1]	Not predictive of response to bevacizumab

[1]Insufficient: The current clinical evidence cannot definitively demonstrate that the biomarker has predictive or prognostic value in metastatic colorectal cancer. EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor.

be detected by fluorescence *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH) or polymerase chain reaction (PCR)-based methods. Interestingly, the EGFR GCN evaluated by quantitative PCR does not appear to correlate with the clinical outcome of patients, whereas the results of FISH analysis appear to be associated with an increase in treatment response[32]. The comparability of these methods and their differential impact on results still needs to be defined. However, EGFR copy number is not used in clinical practice to select patients for treatment, partly due to the lack of standardization of FISH technology and the uncertainty of published clinical cutoff values. Further studies are required to assess the increase in EGFR GCN as a predictive biomarker of response to anti-EGFR therapy.

Increased expression of alternative EGFR ligands, such as amphiregulin and epiregulin, may promote tumor growth *via* an autocrine or paracrine loop that signals through EGFR and have been shown in retrospective studies to be predictive of response to cetuximab[33-35]. The level of sensitivity to cetuximab was shown to be proportional to the intensity of epiregulin and amphiregulin mRNA expression[35-38]. Two studies demonstrated that mCRC patients with *KRAS* wild-type tumors and high amphiregulin and epiregulin mRNA expression were more likely to have disease control with cetuximab treatment[35,37]. In addition to their predictive value, amphiregulin and epiregulin mRNA expression appears to be a useful prognostic marker in KRAS wild-type patients regardless of whether they were receiving anti-EGFR therapy[39]. Low expression of EGFR activating ligands, amphiregulin and epiregulin, was associated with resistance to anti-EGFR therapy and adverse clinical outcome, however, these ligands are not routinely measured in clinical practice and further evaluation of their role is required.

In brief, the predictive value of EGFR expression remains unconvincing in the use of anti-EGFR therapy. Therefore, the focus has shifted to alterations in the key signaling pathway downstream of EGFR, which may drive the growth and progression of CRC and provide an escape mechanism that allows tumors to overcome the pharmacological blockade induced by anti-EGFR mAbs. *KRAS, BRAF, PTEN,* and *PI3KCA* mutations have been highlighted as the mechanisms that activate the EGFR signaling pathway.

KRAS

KRAS belongs to the rat sarcoma virus (ras) gene family of oncogenes which includes *KRAS*, *HRAS*, and *NRAS*. All of these oncogenes when mutated have the ability to transform cells, but KRAS is the most commonly mutated RAS family member in CRC[40]. *KRAS* mutations occur in approximately 35%-45% of mCRC patients, and lead to the constitutive activation of *EGFR* downstream pathways[3]. *KRAS* mutation is thought to be a fairly early event in colon carcinogenesis and appears to be ⩾ 95% concordant between primary tumor and metastatic sites[41-43]. Point mutations in *KRAS* occur most frequently in codons 12, 13 (exon 2), 61 (exon 3)[44], and 146 (exon 4)[45], and up to 90% of activating *KRAS* gene mutations are detected in codons 12 (82%-87%) and 13 (13%-18%). These are generally observed as somatic mutations.

A number of studies have evaluated the potential prognostic role of *KRAS* in CRCs, but the data are conflicting largely due to the differences in methodology and datasets analyzed[46-50]. The first RASCAL meta-analysis evaluated the *KRAS* gene status in 2721 patients, and suggested that the presence of a mutation increased the risk of recurrence ($P < 0.001$) and death ($P = 0.004$)[46]. This finding was later restricted to the *G12V* mutation, which had a statistically significant impact on treatment failure-free survival (HR = 1.3, $P = 0.004$) and OS (HR = 1.29, $P = 0.008$)[47]. Furthermore, the N0147 trial which evaluated the treatment with cetuximab combined with FOLFOX in patients with resected stage Ⅲ CRC showed that the 3-year disease-free survival in patients with wild-type *KRAS* was significantly better than that in

patients with *KRAS* mutations (72.3% *vs* 64.2%, HR = 0.7, $P = 0.004$). These analyses suggest that *KRAS* mutations are independent prognostic factors[51]. The COIN trial assessed the effects of cetuximab combined with oxaliplatin and fluoropyrimidine chemotherapy as first-line treatment in patients with advanced CRC. This trial also showed that a *KRAS* mutation was a strong negative prognostic factor, and the median OS was significantly shorter in patients with *KRAS*, *NRAS*, or *BRAF* mutations ($n = 706$, 13.6 mo) compared to those with wild-type *KRAS, NRAS,* and *BRAF* ($n = 581$, 20.1 mo), irrespective of treatment[52].

However, a recent study by Roth *et al*[53] suggested that the prognostic value of *KRAS* mutation status for PFS and OS was lacking in large adjuvant trials of patients with stage Ⅱ and Ⅲ resected colon cancer. Investigators from the PETACC-3 trial retrospectively analyzed archival tissue ($n = 1564$) for mutations in *KRAS* (exon 2, codons 12 and 13) and found no clear association with relapse-free survival (RFS) or OS in both univariate and multivariate analyses. In the CALGB 89803 study[54], stage Ⅲ CRC patients with *KRAS* mutated tumors did not experience any difference in DFS, RFS and OS rates compared to patients with KRAS wild-type tumors.

In advanced CRC, a few phase 3 studies comparing cetuximab[55] or panitumumab[20,56] with best supportive care (BSC) in the third-line setting demonstrated no significant prognostic value based on *KRAS* mutation status. Two large studies evaluating the addition of cetuximab or panitumumab to chemotherapy and bevacizumab in the first-line setting did not find a prognostic value for *KRAS* mutational status[57,58].

It may be difficult to interpret the various studies published on the prognostic role of KRAS. Therefore, further prospective studies are required to confirm whether a specific *KRAS* mutation might lead to a clinically relevant prognostic effect in patients with CRC.

The predictive value of KRAS has been investigated extensively in the era of EGFR-targeted therapy in colon cancer. Evidence from several clinical trials demonstrated that *KRAS* mutations have emerged as a major predictor of resistance to anti-EGFR mAbs in CRC. Several retrospective analyses have been conducted to explore the role of *KRAS* mutations as a negative predictive biomarker of tumors in patients with mCRC treated with anti-EGFR antibody (with or without chemotherapy)[13,55,59]. The first study to evaluate the correlation between *K-RAS* mutational status and lack of response to treatment with cetuximab was performed by Lièvre *et al*[59]. They analyzed 30 patients predominantly treated with cetuximab plus irinotecan after previous exposure to chemotherapy, and *KRAS* mutations were observed in 13 of the 30 patients enrolled (43%). None of the mutated tumors responded to cetuximab treatment. The OS of KRAS wild-type patients was significantly higher compared to those with mutated *KRAS*. The negative predictive value of *KRAS* mutations for response to anti-EGFR therapy has been confirmed in a number of single arm retrospective studies using the EGFR inhibitors cetuximab or panitumumab alone or in combination with chemotherapy. These retrospective studies revealed that patients with *KRAS* mutations receiving first and subsequent lines of treatment do not benefit from anti-EGFR therapy, and that they show no survival benefit from such treatments[13,59].

Data from phase Ⅲ trials using anti-EGFR targeted therapy in the metastatic setting also suggested that mutated KRAS status predicts a lack of response[20,60,61]. The biomarker analysis of the pivotal phase Ⅲ trial of panitumumab monotherapy in the relapsed or refractory setting was the first large study ($n = 463$ patients) to confirm the negative predictive value of *KRAS* mutations[20]. This study found that in those treated with panitumumab, PFS was 12.3 wk in the subgroup of patients with the wild-type *KRAS* gene, but only 7.4 wk in the subgroup of patients with the mutant *KRAS* gene. This was statistically significant. The PRIME trial evaluated the addition of panitumumab to FOLFOX4 for the initial treatment of patients with KRAS wild-type mCRC[62]. The results were prospectively analyzed by tumor KRAS status, which demonstrated a significantly longer PFS when panitumumab was added to chemotherapy in patients with *KRAS* wild-type tumors (9.6 mo *vs* 8 mo, respectively; HR = 0.80, 95%CI: 0.66-0.97, $P = 0.02$). Furthermore, additional phase Ⅲ studies have shown that only patients with KRAS wild-type CRC will benefit from the addition of panitumumab to FOLFIRI as second-line treatment[63].

Data have recently been published from two large randomized phase Ⅱ-Ⅲ studies carried out to examine the benefits of cetuximab as first-line treatment for mCRC[60,61]. The CRYSTAL study demonstrated that only patients with wild-type KRAS tumors benefited from the addition of cetuximab to FOLFIRI, showing a higher response rate (RR) (57.3% *vs* 39.7%, $P < 0.0001$), longer PFS (median, 9.9 mo *vs* 8.4 mo, $P = 0.012$) and longer OS (median, 23.5 mo *vs* 20.0 mo, $P = 0.0093$). In patients whose tumors carried *KRAS* mutations, there was no evidence of benefit associated with the addition of cetuximab to FOLFIRI. The OPUS trial also showed that the addition of cetuximab to the FOLFOX-4 regimen was only beneficial in the wild-type KRAS subgroup[61]. In KRAS wild-type patients, the addition of cetuximab to FOLFOX induced a significant increase in RR (61% *vs* 37%; $P = 0.011$) and PFS (7.7 mo *vs* 7.2 mo, HR = 0.57, $P = 0.0163$) without OS benefit. In contrast, a negative impact on treatment efficacy was noted when cetuximab was added to chemotherapy in patients with *KRAS* mutant mCRC[64]. These results indicate that *KRAS* mutated patients do not benefit from the addition of cetuximab to conventional chemotherapy.

In contrast to these results, other phase Ⅲ trials found that *KRAS* mutation status was not predictive of benefit when cetuximab was combined with first-line chemotherapy[52,65]. In the NORDIC Ⅶ trial, cetuximab combined with the continuous or intermittent FLOX regimen [bolus 5-fluorouracil (5-FU) plus oxaliplatin] did not significantly improve efficacy compared with FLOX

alone[65]. In the large COIN trial, the addition of cetuximab to oxaliplatin-based chemotherapy did not benefit OS or PFS in KRAS wild-type patients[52].

When anti-EGFR therapy was added to bevacizumab-based first-line chemotherapy in advanced CRC, no additional benefit was observed, even in patients with wild-type KRAS tumors[57,58]. In the CAIRO-2 study, the addition of cetuximab to capecitabine, oxaliplatin, and bevacizumab as first-line treatment in patients with mCRC had no effect on RR (50% *vs* 61.4%; $P = 0.06$) or PFS (median, 10.5 *vs* 10.6; $P = 0.3$) among those with tumors carrying wild-type *KRAS*. Similarly, in the PACCE study, the addition of panitumumab to bevacizumab and oxaliplatin-based chemotherapy was associated with shorter PFS and OS in patients with tumors carrying wild-type *KRAS*. These data suggest a detrimental effect following the addition of antiangiogenic agents to anti-EGFR therapies in advanced CRC.

Based on current information from these clinical trials, the guidelines of the National Comprehensive Cancer Network (NCCN), the ESMO (European Society for Medical Oncology), and the ASCO recommend the use of anti-EGFR-directed therapy only in mCRC patients with wild-type KRAS status. In addition, the NCCN guideline also recommends testing for *KRAS* mutations in codons 12 and 13 in certified laboratories. This is the first true use of personalized medicine in CRC.

However, it is interesting that not all *KRAS* mutations are equal in their biological characteristics and their impact on mediating EGFR resistance. Anecdotal reports indicate that a very small number of patients (< 10%) with KRAS-mutated tumors respond to anti-EGFR therapy[13,66,67] and that about 15% have long-term disease stabilization[68]. Preclinical data demonstrated that cell lines with *KRAS* codon 13 glycine-to-aspartate (*G13D*) mutations exhibit weaker *in vitro* transforming activity than codon 12 mutations[69,70]. Moreover, a recently published retrospective pooled exploratory analysis of patients with chemotherapy-refractory CRC also suggested that patients with p.G13D-mutated tumors showed a trend toward a higher RR than other *KRAS*-mutated tumors. Patients with *KRAS* codon p.G13D mutations who received cetuximab experienced longer PFS and OS compared with BSC alone. In contrast, patients with other *KRAS* mutations did not appear to benefit from cetuximab. Furthermore, benefit from the addition of cetuximab to first-line chemotherapy in patients with *KRAS* p.G13D mutations has also been suggested in a pooled analysis of the CRYSTAL and OPUS studies[71]. Taken together, these data suggest that the use of cetuximab may affect prolonged survival in patients with *KRAS* p.G13D mutations receiving first-line chemotherapy and those with chemotherapy-refractory metastatic colon cancer.

The association between *KRAS* codons 61 and 146 mutations and clinical outcomes in mCRC patients treated with cetuximab has also been investigated[72,73]. It was reported that patients with mCRC that harbors *KRAS* mutations in codons 61 and 146 have a shorter PFS compared to patients with wild-type KRAS and demonstrate resistance to anti-EGFR therapy[73]. In a prospective-retrospective biomarker analysis of the PRIME study, investigators found that not only *KRAS* mutations (mutation at codons 12 or 13) are predictive of treatment resistance to EGFR therapy, but *RAS* mutations (*KRAS* mutation at codons 61, 117 or 146, *NRAS* mutation at codons 12, 13, 61, 117 or 146, and *BRAF* mutations), appear to do the same[74]. These analyses suggest that the assessment of other *RAS* mutations might help optimize the selection of candidate patients for anti-EGFR mAb therapy.

However, our understanding of the biology of KRAS wild-type/mutated genotype and response to anti-EGFR therapy is far from complete. This is underscored by the fact that approximately 40%-60% of mCRC patients with wild-type *KRAS* status fail to respond to anti-EGFR therapy[75]. Moreover, mCRC patients with responsive *KRAS* wild-type tumors inevitably acquire resistance to anti-EGFR therapy and experience tumor progression[76]. A lot of ground remains to be uncovered to clarify the molecular mechanisms that contribute to anti-EGFR therapy resistance/sensitivity, in order that patients can be identified for personalized targeted therapy based on specific genotypes.

BRAF

BRAF, a component of the RAS/RAF/MEK/ERK/MAPK pathway[66], is thought to function as a downstream effector of KRAS. The *BRAF* mutation has been identified in 10%-15% of CRC patients[14,77,78]. The most common *BRAF* mutation in tumors is the V600E mutation, which accounts for 90% of all *BRAF* mutations in CRC. There is an inverse relationship with *KRAS* mutation results, with the V600E *BRAF* mutation seen only in KRAS wild-type tumors[14,73,78]. There is a high concordance in BRAF wild-type status between primary and metastatic tumors, but the level of concordance is lower when the primary tumor harbors a *BRAF* mutation. *BRAF* mutation has been shown to be associated with high grade, right sided tumors, female gender, older age and microsatellite instability high (MSI-H) tumors[53,77,79]. It also has been linked to poor survival in advanced CRC independent of therapy[80].

Recently, a series of studies confirmed the potential adverse prognostic impact of *BRAF* mutations. Yokota *et al*[81] identified *BRAF* V600E mutation as an independent prognostic factor for survival in a representative cohort of 229 patients with mCRC. In this study, *BRAF* mutation was associated with a significantly higher risk of dying from cancer-related causes. This finding is consistent with those of other studies in patients at all disease stages[14,82,83]. In KRAS wild-type patients, BRAF-mutated individuals had a worse outcome in terms of PFS and OS. Furthermore, *BRAF* is a negative prognostic factor for OS, especially in patients with MSI low (MSI-L) and stable (MSI-S) tumors.

In the CRYSTAL-OPUS pooled analysis, patients whose tumors harbored *BRAF* mutations had worse

PFS and OS compared with those who had both KRAS and BRAF wild-type tumors, independent of treatment with cetuximab[84]. These data are consistent with the biomarker analysis of the CAIRO-2 trial[57,85]. This study investigated a large series of patients with mCRC treated with chemotherapy and bevacizumab with or without cetuximab in a subgroup of 520 patients. *BRAF* mutations were detected in 45 (8.7%) tumors and was mutually exclusive of *KRAS* mutations, as reported previously. Patients with BRAF-mutated tumors had a statistically significantly worse PFS and OS compared to patients with wild-type BRAF tumors in both arms of the CAIRO2 trial, however, the RR in the two treatment groups did not differ significantly. The authors concluded that *BRAF* mutations are not restricted to the outcome of cetuximab treatment[85]. These findings further support the hypothesis that *BRAF* mutations are negative prognostic biomarkers.

Several retrospective studies have suggested that the occurrence of *BRAF* V600E mutations accounts for resistance to both cetuximab and panitumumab, but full validation of this association has not been achieved. Di Nicolantonio *et al*[14] retrospectively examined tumors from 113 patients who had received either cetuximab or panitumumab in a second or successive line chemotherapy regimen. None of the BRAF-mutated patients responded to cetuximab or panitumumab, and none of the responders carried *BRAF* mutations. *BRAF*-mutated patients had significantly shorter PFS and OS than wild-type patients. De Roock *et al*[72] reported 4.7% (36 of 761) of *BRAF* mutations in a retrospective pooled study of chemorefractory patients from the European Consortium, and patients with *BRAF* mutations had a significantly lower RR (8.3% *vs* 38% for wild-type; OR, 0.15; $P = 0.0012$), shorter PFS (median, 8 wk *vs* 26 wk for wild-type; HR = 3.74, $P < 0.0001$) and OS (median, 26 wk *vs* 54 wk for wild-type; HR = 3.03, $P < 0.0001$) compared with BRAF wild-type patients. Recently, in the phase Ⅲ PICCOLO trial[86], designed to evaluate the role of panitumumab combined with irinotecan as second or subsequent line therapy for prospectively tested KRAS wild-type advanced CRC, patients with tumors bearing a *BRAF* mutation (13.6%) had a poor prognosis and panitumumab had an adverse effect on survival in this subgroup. These results suggest that wild-type *BRAF* is required for response to anti-EGFR mAb in mCRC. Similarly, Souglakos *et al*[77] assessed the predictive value of *BRAF* mutations in 100 patients treated with cetuximab, including 8 in the first line, 37 in the second, and 55 in the third or higher, always in combination with chemotherapy. No patient with *BRAF* mutations responded to cetuximab. Patients with *BRAF* mutations also had a shorter PFS, regardless of whether cetuximab was administered in the second, third or higher lines.

However, unlike *KRAS* mutations, the negative predictive value of *BRAF* mutations to anti-EGFR therapies in the first-line treatment has not been demonstrated[57,64,84,87]. In the pooled analysis of OPUS and CRYSTAL, patients with *BRAF* mutations seemed to benefit from the addition of cetuximab to first-line chemotherapy with an increase in OS and a doubling of PFS, although these findings did not reach statistical significance, most likely due to the low *BRAF* mutation frequency[84]. This result raises the possibility that the addition of a biological agent might be effective for disease control, at least as first-line chemotherapy, in patients with wild-type KRAS and mutant BRAF. These differences were not statistically significant due to the limited number of patients in this group.

The association between *BRAF* mutations and the efficacy of anti-EGFR therapy remains controversial, but its significant negative prognostic value has been established. Even if the *BRAF* mutation has been shown to be predictive, its low prevalence suggests that it may have limited utility in selecting patients for anti-EGFR therapy in clinical practice. The novel strategy of targeting BRAF kinase is warranted for further treatment of patients with *BRAF* mutations to improve their poor survival.

PIK3CA STATUS

In addition to *KRAS* and *BRAF*, activation of the PI3K signaling pathway can also be oncogenically deregulated either by activating mutations in the *PIK3CA* p110 subunit or by inactivation of the *PTEN* phosphatase. Constitutive activation of the PI3K/AKT pathway has been hypothesized to play an important role in the development of a number of human cancers, including colon cancer. Activating mutations in the *PIK3CA* are described in approximately 10%-20% of unselected CRC patients[48,88-90], mainly in exon 9 or 20. Exons 9 and 20 hotspots exert different biochemical and oncogenic properties. Unlike *BRAF* mutations, *PIK3CA* mutations can co-occur with *KRAS* and *BRAF* mutations[72,91].

Several studies have suggested that *PIK3CA* mutations may be associated with resistance to EGFR mAb therapy[52,92-94]. Preclinical data shows that colon cancer cell lines with activating *PIK3CA* mutations were more resistant to cetuximab than PIK3CA wild-type cell lines. Based on these preclinical data, several retrospective studies have evaluated the predictive value of *PIK3CA* mutations in the clinic. Initial reports show that *PIK3CA* mutations are able to predict resistance to anti-EGFR mAbs in unselected mCRC patients, and more importantly in wild-type KRAS patients whose nonresponse to treatment cannot be predicted by *KRAS* mutations[90,95]. Sartore-Bianchi *et al*[95] found activating *PIK3CA* mutations in 15 (13.6%) of 110 patients treated with cetuximab or panitumumab-based regimens, but none of the *PIK3CA* mutated patients achieved a response to anti-EGFR mAbs, compared with a RR of 23% in 95 patients with wild-type PIK3CA ($P = 0.0337$). Wu *et al*[92] conducted a systematic review and included eight studies which reported survival outcome in 839 mCRC patients. They found that *PIK3CA* mutations were significantly associated with poorer PFS in unselected patients, and observed

a worse OS in KRAS wild-type patients with *PIK3CA* mutations. However, the clinical data regarding *PIK3CA* mutations and response to EGFR mAbs are conflicting. A study by De Roock *et al*[72] found that *PIK3CA* mutations in exon 9 were more common (10% of all samples), but only mutations in exon 20 of *PIK3CA* (3% of all samples) were statistically associated with resistance to cetuximab-based therapy. Importantly, these mutations were also associated with a negative effect on PFS and OS. A meta-analysis by Mao *et al*[93] recently showed that *PIK3CA* mutations, in particular in exon 20, were likely to be related to the prognosis of KRAS wild-type mCRC patients treated with anti-EGFR mAbs. The predictive power of exon 20 mutations was also greater than that of any exon mutations and exon 9 mutations. These findings suggest that exon 20 and exon 9 mutations may differ in their power of predicting the prognosis of mCRC patients. If *KRAS* is unmutated, assessing the *PIK3CA* exon 20 mutations provides additional information on patient outcome.

The predictive value of *PIK3CA* mutation status has been demonstrated, however, the prognostic significance of *PIK3CA* mutations in CRC remains unclear. A number of previous studies have examined the prognostic role of *PIK3CA* mutations in CRC. Recent data suggest that the presence of *PIK3CA* mutations predicts poor prognosis for early stage CRC patients and mCRC patients[95,96]. Patients with *PIK3CA* mutations were more likely to experience local recurrences than patients without mutations[96]. In a study of 586 patients by Barault *et al*[48], it was found that mutations of at least one gene among *KRAS*, *BRAF* and *PIK3CA* were associated with a lower 3-year survival rate. Kato and coworkers carried out an analysis of 158 CRC tissue samples and identified *PIK3CA* mutations as the only independent and significant prognostic factor for worse RFS in stage Ⅱ/Ⅲ CRC patients[97]. These results are in contrast with those observed in the metastatic setting. Cappuzzo *et al*[98] described a *PIK3CA* mutation in 17.7% (14/85) of cetuximab-treated mCRC patients, but found no difference in overall response rate (ORR), time to progression (TTP) and OS compared to the wild-type population. Liao *et al*[99] analyzed *PIK3CA* pyrosequencing in 1267 CRC patients, and *PIK3CA* mutations were detected in 189 (16%) of 1170 cases. The results showed that concomitant *PIK3CA* mutations of both exons 9 and 20 were associated with a poorer prognosis. In contrast, neither *PIK3CA* exon 9 mutation nor exon 20 mutation alone appeared to have substantial prognostic influence.

Taken together, these findings are not uniform and there are contradictory reports, thus it is not anticipated that in the short-term future *PIK3CA* mutation testing will be performed in routine clinical practice to determine eligibility for anti-EGFR antibody therapy. It is also estimated that only 3%-10% of patients who are in the KRAS wild-type group will have a *PIK3CA* mutation, therefore the potential contribution of this mutation for individualized treatment of CRC will be limited. Thus, further evidence from large randomized clinical trials and standardization of analysis will be required to establish a role for these genetic markers in mCRC treatment.

PTEN STATUS

PTEN is the only tumor suppressor gene involved in the PI3K-AKT-mTOR pathway. It has been shown that inactivation of *PTEN* phosphatase deregulates the PI3K pathway. *PTEN* loss is observed in 20%-40% of CRC tumors[94,100], and it has been found to co-occur with *KRAS*, *BRAF* and *PIK3CA* mutations[91,101]. *PTEN* expression shows only approximately 60% concordance between primary tumor and distant metastases[40,94]. Loss of *PTEN* expression is associated with aggressive CRC and lack of benefit with cetuximab in patients with chemotherapy-refractory mCRC. It may provide valuable prognostic and predictive information to aid treatment strategies for patients[94].

The prognostic role of *PTEN* in CRC is still under investigation, and inconclusive results have been reported. In a retrospective analysis of archival tumor tissue from 173 patients with mCRC, loss of *PTEN* expression (19.9% cases) detected by IHC was associated with inferior OS in a multivariate analysis (HR = 1.9, 95%CI: 1.1-3.2, $P = 0.026$)[100].

PTEN also shows promise as a predictive marker for wild-type *KRAS* patients treated with an anti-EGFR-based regimen[102,103]. Wang *et al*[102] analyzed *PTEN* expression in 852 mCRC patients treated with anti-EGFR mAbs, and loss of *PTEN* expression was detected in 242 (28.4%) patients. Anti-EGFR mAb therapy resulted in improved PFS and OS in patients unselected by *KRAS* mutation with normal *PTEN* expression over loss of *PTEN* expression. Better PFS and OS were observed in wild-type KRAS patients with normal *PTEN* expression *vs* loss of expression. Razis *et al*[103] reported that normal PTEN protein expression was associated with a higher RR and longer TTP in patients treated with cetuximab-based therapy, despite a 50% RR observed in patients who had lost PTEN protein expression. These data showed that loss of *PTEN* expression is a potential biomarker for resistance to anti-EGFR mAb therapy, particularly in mCRC patients with KRAS wild-type tumors. Interestingly, preserved *PTEN* expression in metastatic samples was predictive of response to cetuximab, while this was not observed in primary tumor tissue with preserved *PTEN* expression. Therefore, these data are limited and should be considered exploratory. The value of *PTEN* as a predictive or prognostic marker in mCRC cannot be established yet.

OTHER POTENTIAL BIOMARKERS

HER2 gene status

In contrast to gastric and breast cancer, human epidermal growth factor receptor 2 (HER2) protein overexpression and *HER2* gene amplification are relatively rare in CRC.

Some studies have shown that *HER2* gene amplification was significantly related to resistance to cetuximab or panitumumab and was associated with a significantly worse PFS and a trend towards a worse OS[104-106]. However, other studies have not found a predictive or prognostic role for HER2[85,107]. Recently, data from a retrospective study have suggested that HER2 status detected by FISH might represent an additional useful marker for the identification of advanced CRC patients who may benefit from anti-EGFR targeted therapies[105,106]. A total of 407 chemorefractory mCRC patients treated with cetuximab alone or in combination with irinotecan were evaluated and *KRAS* and *BRAF* mutations were assessed. The status of the *HER2* gene was evaluated in 288 cases. Interestingly, *HER2* gene-positive patients had a significantly higher RR, longer PFS and OS compared with *HER2* gene-negative patients, but when cases were stratified according to *KRAS* and *BRAF* mutations, no significant differences in RR, PFS and OS were observed between *HER2*-positive and negative cases. In conclusion, the interplay between *EGFR* and *HER2* requires further investigation for future best-tailored treatments.

c-Met and insulin-like growth factor receptor 1 pathways

MET, the hepatocyte growth factor receptor, is a receptor tyrosine kinase (RTK) involved in cellular proliferation and apoptosis. The activation of MET may lead to the activation of pathways downstream of RAS, such as Raf/MEK/MAPK and the PI3K/protein kinase B pathway (PKB). In addition, MET is able to directly activate the PI3K/PKB pathway in a RAS-independent manner[108]. Several preclinical findings suggest that MET can interfere with anti-EGFR strategies. Inno *et al*[109] recently reported that compared with low/normal expression, c-Met overexpression significantly correlated with shorter median PFS and median OS in 73 patients with mCRC treated with cetuximab-containing regimens. Cappuzzo *et al*[29] also assessed MET at the genomic level using FISH in 85 EGFR FISH-positive mCRC patients treated with cetuximab. Both patients with MET amplification responded to cetuximab therapy, although the number of patients was too low to draw any conclusion.

Insulin-like growth factor receptor 1 (IGF1R) is also a transmembrane RTK implicated in promoting oncogenic transformation, growth and survival of cancer cells. IGF1R is overexpressed in 50%-90% of CRCs[110], and preclinical studies suggest that this target results in upregulation in the majority of CRC patients, poor prognosis and resistance to anti-EGFR strategies[111].

P53 mutations

TP53 is a tumor-suppressor gene located on chromosome 17p, and mutations in this gene occur in about half of CRCs. A large number of studies have described the effects of genetic TP53 alterations on progression and outcome of CRC, and the results are heterogeneous and conflicting. Most studies which showed an association between TP53 alterations and worse outcome employed IHC and the remainder employed DNA analysis. Therefore, it is likely that activation of the EGFR pathway will contribute to cancer and anti-EGFR antibodies will be efficient in tumors only if TP53 is inactivated. Based on these observations, Oden-Gangloff *et al*[112] evaluated the combined impact of KRAS and TP53 status on clinical outcome in 64 mCRC patients treated with cetuximab-based chemotherapy, and suggested that *TP53* mutations are predictive of cetuximab sensitivity.

In conclusion, these data suggest that *TP53* genotyping could have an additional value in mCRC patients without *KRAS* mutations to optimize the selection of patients who could benefit from anti-EGFR therapies. The clinical relevance of these results should be confirmed in larger mCRC series.

Angiogenesis

Angiogenesis has become a major target in CRC therapy. A variety of anti-angiogenesis approaches have been evaluated for the treatment of mCRC. Bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor A (VEGF-A), is approved for first-line treatment of mCRC. Novel antiangiogenic drugs, such as regorafenib (a novel tyrosine kinase inhibitor targeting VEGFR, PDGFR, FGFR, RET, KIT and TIE2) and aflibercept (a VEGF trap), have also been licensed by the United States Food and Drug Administration based on trials showing modest improvements in OS[113,114]. However, despite the increasing use of various antiangiogenic drugs and intense research efforts, there is a lack of evidence for validated biomarkers in terms of response to antiangiogenic therapy. Several markers that have appeared promising in preclinical models have failed as predictors of response in human trials (Table 2)[115-117]. To date, no biomarkers have emerged that are capable of predicting the efficacy of these agents.

Several recent studies on the identification of predictive biomarkers for bevacizumab have been performed. In the pivotal AVF2107 study of bevacizumab added to chemotherapy in the first-line setting of advanced CRC, plasma VEGF levels, primary tumor tissue VEGF expression, microvessel density and genotypic characteristics of the malignant cells such as *KRAS*, *BRAF*, *TP53* mutations, and *TP53* overexpression were evaluated, but none had predictive value for bevacizumab activity. These findings were recently confirmed in the MAX trial, in which the *KRAS* and *BRAF* mutation status failed to predict benefit with bevacizumab[118-120]. In this study, the expression levels of *VEGF* family members A through D and VEGF receptors, VEGFR-1 and VEGFR-2, were also analyzed using IHC, and the results showed that VEGF-D expression was a predictor of response to bevacizumab treatment. For patients treated with bevacizumab, low VEGF-D expression was predictive of a significantly longer PFS and OS interval than those in patients with high levels of VEGF-D expression. In the NO16966 trial[119], exploratory analyses found that high CD31, high VEGF-A, and low EGFR-2 expression lev-

Table 2 Summary of key biomarkers investigated in clinical trials of bevacizumab

Key biomarkers evaluated
KRAS mutational status
BRAF mutational status
p53 mutational status
VEGF and *VEGFR-2* (*KDR*) gene expression
VEGF A- to VEGF-D, VEGFR-1, and VEGFR-2 protein expression
CD31 expression
Neuropilin expression
Stromal thrombospondin-2 expression
Microvessel density
Plasma VEGF levels

VEGF: Vascular endothelial growth factor.

els were correlated with a longer duration of response, and high levels of neuropilin and placental growth factor were associated with less benefit from bevacizumab. However, these results are considered exploratory and need to be confirmed in additional clinical trials.

Blood-based biomarkers have, until now, produced mixed results. Several studies have demonstrated that plasma VEGF-A is a prognostic biomarker in CRC, but it is unable to predict response to antiangiogenic treatment in mCRC[121,122]. A retrospective analysis of 1816 patients with colon, renal cell, and lung cancer found that plasma VEGF levels were not predictive of benefit from bevacizumab[123]. However, an association between plasma VEGF and benefit from bevacizumab treatment was observed in a breast cancer trial[124]. Further prospective studies are underway to validate the value of plasma VEGF-A in clinical practice. VEGF polymorphisms are also potentially promising biomarkers, however, it is not currently possible to personalize treatment with antiangiogenic therapies[125].

More recently, preclinical data supporting the role of fibroblast growth factor receptor (FGFR) and platelet-derived growth factor receptor (PDGFR) signaling in angiogenesis have been reported. Inhibition of these pathways holds potential therapeutic benefit for cancer patients[126]. In addition, one or both of these pathways have been associated with resistance to agents targeting the EGFR and VEGF[127]. Some studies have elucidated the role of FGFR and PDGFR in colon cancer angiogenesis. However, only a few studies have analyzed the clinical implications of FGFR/PDGFR expression in CRC. Wehler *et al*[128] in a series of 99 human colorectal carcinomas, reported that coexpression of PDGFRα/β observed in 57% of tumor samples, was significantly associated with lymphatic metastasis ($P = 0.007$) and advanced tumor stage ($P = 0.03$). Schimanski *et al*[129] reported that specific receptor tyrosine kinases (TK) were overexpressed in *KRAS*-mutated CRC. In a study by Nakamura *et al*[130], patients with high PDGF-BB expression had a significantly poorer survival rate than those with low PDGF-BB expression. A multivariate analysis also demonstrated that PDGFR expression was an independent prognostic factor. Sato *et al*[131] reported that overexpression of the *FGFR1* gene leads to liver metastasis in CRC. Matsuda *et al*[132] also found overexpression of the *FGFR2*, both *FGFR2IIIc* and *FGFR2IIIb*, in colorectal carcinomas which tended to correlate with distant metastasis. On the other hand, *FGFR2IIIb* expression in colorectal carcinomas did not correlate with survival or metastasis[133]. It was also found[134] that in colorectal carcinoma cases, expression levels of *FGFR2IIIc* in tumor cells were correlated with advanced carcinogenesis stages. Furthermore, *FGFR2IIIc* expression correlated with metastasis and poor prognosis of colorectal carcinomas, which suggested that *FGFR2IIIc* may have a potential use in colorectal carcinoma therapy. A number of agents that target FGF and/or PDGF signaling are now in development for the treatment of mCRC. Potential predictive biomarkers for these pathways are being investigated, but none have been validated for clinical use. Whether this could translate into a higher likelihood of responding to PDGFR/FGFR targeted agents is a matter of speculation.

Hypertension is a common adverse effect of anti-VEGF therapy. The development of hypertension due to anti-VEGF treatment has also been evaluated as a predictive biomarker. An increase in blood pressure may reflect successful inhibition of the VEGF pathway. However, the role of hypertension in predicting responsiveness to antiangiogenic drugs is controversial. In the AVF2107 study, the development of hypertension predicted better PFS (HR = 0.55, $P = 0.0008$) and better OS (HR = 0.43, $P = 0.0001$), but this was not confirmed by other studies[135]. The role of hypertension as a predictive biomarker requires further evaluation, particularly as it is standard practice to treat hypertension as soon as it develops[136].

Epigenetics in CRC

Epigenetics describe the changes in phenotype or gene expression that do not involve DNA sequence changes. CRC is considered a genetic disease with the histologic progression of carcinogenesis characterized by sequential genetic and epigenetic alterations[137]. Epigenetic instability in CRC is manifested in a variety of ways including hypermethylation of gene promoters that contain CpG islands and global DNA hypomethylation. The role of epigenetics in CRC development and pathogenesis is beginning to be defined. Retrospective studies have proposed candidate markers, such as CpG island methylation (CIMP), which may predict poor outcome for CRC patients after fluorouracil treatment[138]. However, there are conflicting results and studies are required to determine the reproducibility of the data[139]. Promoter CpG island methylation of the Werner syndrome gene[140] and the UDP-glucuronosyl-transferase gene, *UGT1A1*[141], have been reported to influence the effects of and response to the topoisomerase inhibitor, irinotecan, with these studies being directly related to silencing of genes involved in the mechanism of action of this drug. However, the data are not currently robust enough to recommend its clinical use[142,143].

Epigenetic changes in CRC are also potential mark-

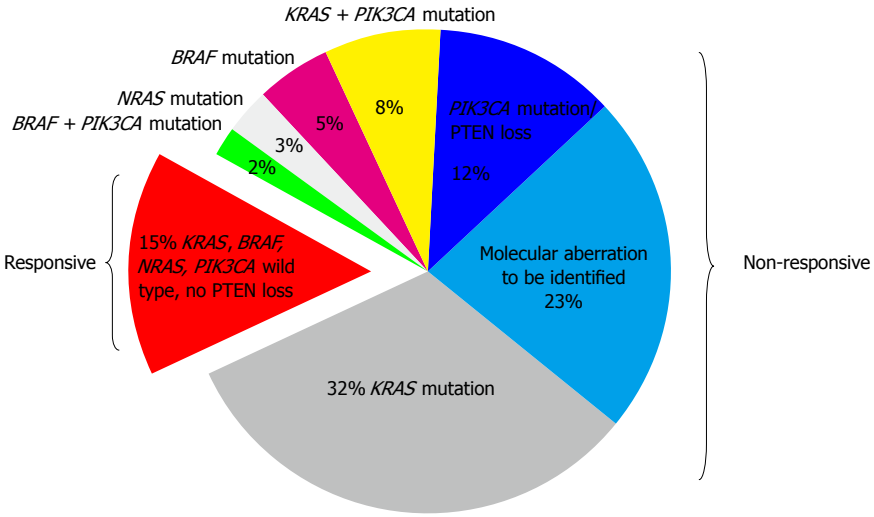

Figure 1 Prevalence of epidermal growth factor receptor pathway deregulations and response to monoclonal antibodies targeting epidermal growth factor receptor in chemotherapy-refractory advanced colorectal cancer.

ers for the early detection of CRC and prediction of prognosis. Several publications report a prognostic role for promoter CIMP markers, such as $p16^{INK4A}$, $p14^{ARF}$, *MGMT*, *HPP1*, *HLTF*, and *ID4*, but their effects seem to be dependent on the presence of other methylated markers or adjuvant treatment[139]. A prognostic role was also suggested for CIMP, and a worse prognosis for patients with CIMP CRCs was observed in most studies, although conflicting results have also been reported[140]. These examples of the potential prognostic use of alterations in DNA methylation highlight the need for validation of their clinical utility in observational, population-based studies to assess the natural course of the disease.

Despite these examples and other studies of predictive and prognostic epigenetic markers in CRC, none have yet been developed to the point of clinical utility. Continued efforts to investigate these molecular mechanisms will allow for a better understanding of the role of epigenetic alterations in CRC and will lead to the translation of these insights into the clinical arena.

CONCLUSION

Currently, the treatment of advanced CRC varies and oncologists face complicated decisions in the selection of the most appropriate treatment options for their patients. Predictive and prognostic biomarkers can facilitate clinical decision-making and are becoming increasingly important with the development of targeted therapies for advanced CRC. The identification of molecular biomarkers that have predictive and/or prognostic significance in CRC is essential to improve anti-cancer treatments and patient outcome[144]. Several molecular biomarkers have been studied over the past two decades and encouraging improvements have been achieved. However, the results of published studies have often been conflicting and several drawbacks affect the reliability of conclusions[145]. First, most published studies were retrospective analyses of a single marker or included a small sample size. These study designs are unlikely to accurately predict disease progression with sufficient resolution and reproducibility. Second, data analysis and interpretation still remain challenging, although many advances have been made in technologies for profiling and in decreasing the requirements of the input material. The data from current studies usually lack definition, adequate validation, and cannot be used in clinical practice for decision-making. Furthermore, the lack of methodology standardization involved in the detection of biomarkers, the lack of comprehensive analysis of a particular molecular pathway, and incomplete analysis of biomarkers have all contributed to the frustration associated with biomarker validation. Therefore, to date, only *KRAS* gene has entered routine clinical practice as a predictive marker of response to EGFR-targeted therapies in advanced CRC.

A number of comprehensive biomarker-driven studies are currently underway. *BRAF* V600E mutation is prognostic of patient outcome with respect to survival, but not clearly predictive of treatment effects with anti-EGFR agents in patients with mCRC. The low prevalence of such mutations makes it difficult to evaluate these mutations as predictive biomarkers in clinical practice. The predictive and prognostic value of *PIK3CA* mutation, *PTEN* deletion and *TP53* mutation is presently under evaluation, but clinicians are currently unable to use these data in clinical practice for decision-making. In the future, *NRAS*, *PIK3CA* and *PTEN* status may be useful when combined with *KRAS* and *BRAF* mutation analysis to predict which mCRC patients will benefit from anti-EGFR therapy (Figure 1). The identification of a biomarker to predict response to anti-VEGF agents is lacking, and further data are required from large well designed prospective studies to understand the biological processes underlying response and/or resistance. Novel prospective randomized controlled trials are needed to determine the role of various putative molecular markers,

and hopefully this will facilitate the development of personalized therapy based on the molecular profile of CRC.

In addition to these molecular markers, many patient-related factors may also influence response to targeted therapy, including age, sex, tumor subtype, disease stage, comorbid diseases, overall PS, pharmacokinetic, pharmacodynamic and pharmacogenetic factors. These factors should be considered as important predictive and prognostic biomarkers in CRC.

In the future, it is anticipated that new biomarkers will be developed that can further personalize the treatment of this important human cancer. In the era of targeted therapies, it is further anticipated that new small molecule drugs that target specific gene mutations (for example, *BRAF* inhibitors) and genetic translocations will be developed in association with specific biomarker tests that are linked to drug response and patient eligibility for treatment.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Gastric cancer in Africa: Current management and outcomes

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Abstract

Gastric cancer is the fourth most common cancer and second most common cause of cancer death worldwide. Globally, gastric cancer poses a significant public health burden - both economically and socially. In 2008, the economic burden from premature cancer deaths and disability was $895 billion and gastric cancer was the second highest cancer responsible for healthy life lost. With the expected increase in cancer deaths and non-communicable diseases, these costs are expected to rise and impact patient care. World Health Organization, estimates a 15% increase in non-communicable disease worldwide, with more than 20% increase occurring in Africa between 2010 and 2020. Mali, West Africa, is ranked 15th highest incidence of gastric cancer worldwide at a rate of 20.3/100000, yet very scarce published data evaluating etiology, prevention or management exist. It is understood that risk factors of gastric cancer are multifactorial and include infectious agents (*Helicobacter pylori*, Epstein-Barr virus), genetic, dietary, and environmental factors (alcohol, smoking). Interestingly, African patients with gastric cancer are younger, in their 3rd-4th decade, and present at a late stage of the disease. There is sparse data regarding gastric cancer in Africa due to lack of data collection and under-reporting, which impacts incidence and mortality rates. Currently, GLOBOCAN, an International Agency for Research on Cancer resource, is the most comprehensive available resource allowing comparison between nations. In resource limited settings, with already restricted healthcare funding, data is needed to establish programs in Africa that increase gastric cancer awareness, curtail the economic burden, and improve patient management and survival outcomes.

Key words: Gastric cancer; Africa; Cancer outcomes; Cancer survival; Cancer management; Non-communicable diseases

Core tip: There is a paucity of published data regarding gastric cancer in Africa and a need for more research to elucidate etiology and management. There is a growing opportunity for partnerships between African nations and more developed nations to advance the understanding and management of gastric cancer and thus improve overall patient outcomes. Such partnerships would provide a bilateral learning opportunity and help set a platform for training opportunity amongst healthcare providers.

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INTRODUCTION

Gastric cancer is the fourth most common cancer worldwide, and second most common cause of cancer death[1]. In 2008, the economic burden from premature cancer deaths and disability was $895 billion and gastric cancer

was the second highest cancer responsible for healthy life lost[2]. With the expected increase in cancer deaths and non-communicable diseases, these costs are expected to rise and impact patient care. World Health Organization (WHO), estimates a 15% increase in non-communicable disease worldwide, with more than 20% increase occurring in Africa between 2010 and 2020[3].

In Africa, gastric cancer is ranked twelfth most common cancer[1]. The estimated incidence and mortality rates of gastric cancer in Africa is 4/100000 and 3.8/100000 respectively[1]. The incidence is higher in men (4.7/100000) than in women (3.3/100000), as is mortality rate at 4.5/100000 and 3.2/100000 respectively[1]. There is a great deal of variation in reported incidence and mortality within the individual African countries. For instance, in Mali, West Africa, gastric cancer is the most common cancer in men with an incidence rate 21.6/100000 and mortality rate 21.1/100000[1]. These rates are markedly higher than developed nations such as United States/United Kingdom, yet very scarce published data evaluating etiology, prevention or management exist[4]. The lack of published reports may have to do with the focus on communicable diseases and less attention to non-communicable disease (NCDs). The variation in reported incidence and mortality within the individual African countries is most likely related to etiology. Recognized carcinogens include diet, infectious agents, inflammatory process or genetic variability[5]. Low incidence and mortality rates reported for some regions may be due to the limited diagnostic capability and inadequate data recording. Interestingly, gastric cancer in African patients are younger, in their 3rd-4th decade[6], and present at a late stage of the disease[7].

It has been recognized that there is an upward trend in NCDs, including cancers within developing nations[8]. WHO estimates an increase in cancer deaths of 45% between the years 2007 to 2030[9]. Cancer kills more people than AIDS, tuberculosis and Malaria combined[10], yet it has received less focus. Understanding risks, etiology and management of gastric cancer impacts the survival outcome. With the increasing westernization of diet, obesity, lifestyle (alcohol and smoking), there is increased risk of gastric carcinogenesis. It is prudent to increase research related to cancer in Africa, including gastric cancer.

INCIDENCE AND MORTALITY RATES

A comprehensive and comparable resource for incidence and mortality rates is the GLOBOCAN database. The Globocan database is part of the International Agency of Research on Cancer-World Health Organization (IARC-WHO)[1]. Globocan database is one of the world's largest, most reliable and organized incidence and mortality data resources for gastric cancer in Africa, however, it has its limitations[1]. Globocan recognizes the limitations in data collection. First, the development of the database relies on the cancer registries with the individual countries, which may not capture the realities or may be an under-representation of the incidence and mortality rates[1]. Second, some countries do not have a cancer registry thus reliance on data from individual hospitals[1].

There is significant variation from country to country in Africa with estimated incidence rates as high as 20.3/100000 in Mali to as low as 0.3/100000 in Botswana[1]. Table 1 details the incidence and mortality rates in Northern, Eastern, Central, Southern and Western Africa. The geographic demarcation is that from the Globocan database recognizing 53 African countries, with the highest incidence and mortality from Eastern Africa 4.7/100000 and 4.6/100000 respectively[1]. Other countries with high incidence and mortality rates are La Reunion (12.6/100000, 9.3/100000), Western Sahara (11.9/100000, 11.9/100000), Burundi (9.8/100000, 8.4/100000), Uganda (9/100000, 8.7/100000), Rwanda (8.3/100000, 8/100000), Kenya (7.6/100000, 7.3/100000) and Democratic Republic of Congo (7.3/100000, 7.2/100000)[1]. These rates are higher than Western nations such as United States (4.1/100000, 2/100000) and United Kingdom (5.5/100000, 2/100000), but lower than Eastern nations such as Japan (31.1/100000, 13.5/100000), South Korea (62.2/100000, 22.5/100000) and China (29.9/100000, 22.3/100000). Some African countries with lower incidence and mortality rates are Botswana (0.3/100000, 0.3/100000), Namibia (1.1/100000, 1.1/100000), Lesotho (1.3/100000, 1.3/100000), Equatorial Guinea (1.6/100000, 1.6/100000), Sudan (1.6/100000, 1.8/10000) Malawi (1.7/100000, 1.6/100000), Central Republic of Africa (1.9/100000, 1.8/100000) Swaziland (1.9/100000, 1.9/100000) and The Gambia (1.9/100000, 1.9/100000)[1].

This variation in reporting may be related to the late patient presentation and lack of cancer reporting systems. It is also unclear if these are autopsy reports or live cases. Some of the differences in the rates can be attributed to the degree of advances in medicine within the country and available expertise. McFarlane *et al*[11] conducted a retrospective survey in the Eastern part of Kenya with a population of approximately 1.2 million. The researchers reviewed medical records between 1991-1993, and compared to data from the same area obtained in the 1970s. There was a 10 fold increase in the incidence rate between 1965-1970 and 1991-1993. The authors note that endoscopy services were established in the Eastern part of Kenya in 1980s, which could have impacted diagnostic capabilities. Similar finding of increased gastric cancer incidence over decades in Uganda has been documented but attributed to an increased access to healthcare and endoscopy availability[11]. In Uganda, East Africa, most recent data shows a seven fold increase incidence of gastric cancer from 0.8/100000 in the 1960s to 5.6/100000[12]. This has led to development and implementation of guidelines regarding management of patients presenting with dyspepsia[12].

MANAGEMENT AND OUTCOMES

Initial diagnosis of gastric cancer is typically via an upper endoscopy with biopsy of an abnormal appearing lesion

Table 1 Gastric cancer incidence and mortality rates

	Incidence[1]			Mortality[1]		
	Males	Females	Both	Males	Females	Both
Africa[2]	4.7	3.3	4.0	4.4	3.2	3.8
Northern	3.9	2.4	3.2	3.7	2.3	3.0
Eastern	5.6	4.0	4.7	5.4	3.8	4.6
Central	5.3	4.7	4.0	5.2	4.6	4.8
Southern	4.1	2.2	3.0	3.9	1.8	2.8
Western	4.5	3.3	3.8	4.3	3.1	3.7
Asia	25.9	11.7	18.5	18.3	8.9	13.4
North America	5.8	2.8	4.2	2.8	1.8	2.1
South America	17.3	8.4	12.4	14.2	6.9	10.2
Europe	14.5	7.0	10.3	11.3	5.3	7.9
Australia	7.4	3.4	5.3	4.3	2.1	3.1

[1]Incidence and mortality are represented as age-standardized rates (per 100000); [2]Countries within each geographic region (per GLOBOCAN) is listed below: Africa: Northern: Algeria, Egypt, Libya, Morocco, Sudan, Tunisia, Western Sahara; Eastern: Burundi, Comoros, Djibouti, Eritrea, La Reunion-France, Kenya, Madagascar, Malawi, Mauritius, Mozambique, Rwanda, Somalia, Tanzania, Uganda, Zambia, Zimbabwe; Central: Angola, Cameroon, Central African Republic, Change, Democratic Republic of Congo, Republic of Congo, Equatorial Guinea, Gabon; Southern: Botswana, Lesotho, Namibia, South African Republic, Swaziland; Western: Benin, Burkina Faso, Cape Verde, Cote d'Ivoire, the Gambia, Ghana, Guinea-Bissau, Guinea, Liberia, Mali, Mauritania, Niger, Nigeria, Senegal, Sierra Leone, Togo; Asia: Eastern: China, Japan, South Korea, Mongolia, Taiwan; South-Eastern: Brunei Darussalam, Cambodia, Indonesia, Lao, Malaysia, Myanmar, Philippines, Singapore, Thailand, Timore-Leste, Vietnam; South-Central: Afghanistan, Bangladesh, Bhutan, India, Iran, Kazakhstan, Kyrgyzstan, Maldives, Nepal, Pakistan, Sri Lanka, Tajikistan, Turkmenistan, Uzbekistan; Western: Armenia, Azerbaijan, Bahrain, Gaza Strip and West Bank (Palestine), Iraq, Israel, Jordan, Kuwait, Lebanon, Oman, Qatar, Saudi Arabia, Syria, Turkey, United Arab Emirates, Yemen;North America: United States, Canada; South America: Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, French Guyana, Guyana, Paraguay, Peru, Suriname, Uruguay, Venezuela; Europe: Central and Eastern: Belarus, Bulgaria, Czech Republic, Hungary, Republic of Moldova, Poland, Romania, Russia, Slovakia, Ukraine; Northern: Denmark, Estonia, Finland, Iceland, Ireland, Latvia, Lithuania, Norway, Sweden, United Kingdom; Southern: Albania, Bosnia, Croatia, Cyprus, Greece, Italy, Former Yugoslav Republic of Macedonia, Malta, Montenegro, Portugal, Serbia, Slovenia, Spain; Western: Austria, Belgium, France, Germany, Luxembourg, The Netherlands, Switzerland.

confirmed by histopathology. Staging options include endoscopic (endoscopic ultrasound, EUS) and radiographic [computed tomography (CT), magnetic resonance imaging, positron emission tomography and abdominal ultrasound], with CT being the most common[13]. EUS is not an option in most African countries due to the absence of technology and trained personnel, thus radiology is the mainstay of staging. There are also limitations to staging related to patient financial constraints, which impacts overall patient care. Screening modalities (barium study, gastroscopy and serum pepsinogen) have been implemented in high risk Asian countries such as Japan, South Korea, and Taiwan[14], however these are not part of the management of patients at risk for gastric cancer in Africa. Late presentation plays a significant role in the poor outcome in most African countries; hence increased awareness amongst the general population and more training of healthcare providers have an important role.

After the initial diagnosis of gastric cancer, staging should be performed to determine next step in management. The staging system evaluates nodal and other organ involvement. Staging extends from 0 to 4, with 0 being localized (also known as carcinoma *in situ*) and stage Ⅳ representing metastatic disease to distant organs in the body. This staging has implications on the overall prognosis and treatment plan. With surgical intervention, the 5 year survival outcomes for patients with localized disease are estimated to be 75%, and for those with lymph node involvement the prognosis is poorer, 10%-30%[13]. Factors impacting outcome can be broadly defined as tumor specific or patient specific. The tumour related risk factors are: stage, grade, size, gastric location of primary tumour, lymph node or vascular involvement[13]. The patient specific factors are age, gender, co-morbidities and performance status[13]. Delayed presentation, limited radiotherapy and surgical capability also impact management[7].

Surgery plays a role in diagnosis, palliative therapy or curative therapy[12]. The role of surgery as curative modality seems to be limited to patients who present in the early stage[12,13]. Mabula *et al*[15] conducted a retrospective analysis of gastric cancer patients in Kenya, East Africa, evaluating the clinicopathology and outcome over a 4 year period. They enrolled 232 patients, median age was 52, 92% presented in late stage of the disease, 223 (96%) underwent surgery (commonly gastrojejunostomy) as primary treatment, 56 (24%) chemotherapy and 12 (5.1%) radiotherapy[15]. The post-operative complication and mortality was 37.1% and 18.1% respectively[15]. The 5 year survival was 32.8%[14]. Ahmed *et al*[16] conducted a retrospective study in Nigeria, West Africa, evaluating clinical pattern, management and outcome of 179 patients. The mean age was 51 ± 6 years, 155 (86%) underwent surgical intervention, (mainly gastrectomies), the overall median survival was 13.6 mo[16]. For patients who underwent gastrectomy, the one and five year survival were 70.1% and 21.8% respectively[16]. The post-operative complication and mortality were 43 (27.7%) and 25 (16.1%) respectively[16]. These studies also highlight significant post-operative complications and mortality, which seem related to the late patient presentation. Table 2 summarizes the treatment, post surgical complications and outcomes of gastric cancer in some African countries.

CONCLUSION

Published data regarding gastric cancer management and outcome within most African countries is scarce. It is plausible that overall incidence and mortality in some nations is lower due to the underreporting and lack of an organized, adequate database. What's clear is the consistency of poor prognosis and lower overall survival rates which are related to advanced stage at diagnosis, lack of disease awareness and limited health access. The increase post-operative mortality could be related to the late/advanced stage at presentation, poor performance status of the patient, and other co-morbidities such as non-communicable disease. The healthcare focus has predominantly

Table 2 Treatment and survival outcome

Country	Year of publication and study design	Author	Sample size	Overall survival	Treatment	Post-operative complication	Post-operative mortality	Incidence and Mortality rate per Globocan
Tanzania	2012, retrospective review	Mabula *et al*[15]	232	5 yr survival: 32.8%	Surgery: 223/232 (96.1%) Chemotherapy: 56 (24%) Radiotherapy: 12 (5.1%)	37.1%	18.1%	2/100000 2/100000
Mali (Abstract, French article)	2012	Dembele*et al*[17]	425	1 yr survival: 15.5%	200 (65%): surgery 105 (34.3%) : no surgery 4 (1.3%): chemotherapy	-	-	20.3/100000 19.7/100000
Nigeria	2011, retrospective review	Ahmed *et al*[16]	179	13.6 mo 1 yr post gastrectomy: 70.1% 5 yr post gastrectomy: 21.8%	Surgery: 155 (86.6%)	43 (27.7%)	25 (16.1%)	2.2/100000 2.1/100000
Zimbabwe	2011	Chokunonga *et al*[18]	-	5 yr survival: 20%	-	-	-	5.3/100000 5/100000
Nigeria	2010, retrospective	Osime *et al*[19]	-	mortality rate 39.1%; 66% died within one year of diagnosis	30.4% presented within in year			2.2/100000 2.1/100000
Tunisia (Abstract, French article)	2006, retrospective	Arfaoui*et al*[24]	140	26.5 mo, 13 mo and 5 mo after curative resection, palliative resection and without resection	Curative and palliative resection			
Tunisia (Abstract, French article)	2004	Ayite *et al*[23]	63	3 mo: 21% 1 yr survival: 7%				4.2/10000000 3.9/100000
Senegal (Abstract, French article)	2003	Fall *et al*[20]	60	Total overall survival : 20%	-	-	-	6.1/100000 5.9/100000
Uganda	2001, prospective, descriptive	Ibingira[21]	35	1 yr survival after partial gastrectomy: 39.1% -	94.5% presented with advanced cancer, and no curative surgery possible			9/100000 8.7/100000
Ethiopia	2000, prospective	Johnson *et al*[22]	96		90% presented in advanced stage; 40% had resectable lesions	-	18.6%	3.5/100000 3.4/100000

been in communicable diseases, however recent data show an increasing trend of non-communicable diseases such as obesity, hypertension and cardiovascular diseases which impact both the risk and management of gastric cancer in Africa. More research into gastric cancer management and outcomes in Africa, along with training of healthcare providers to improve patient survival is needed.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Current role of minimally invasive approaches in the treatment of early gastric cancer

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Abstract

Despite declining incidence, gastric cancer remains one of the most common cancers worldwide. Early detection in population-based screening programs has increased the number of cases of early gastric cancer, representing approximately 50% of newly detected gastric cancer cases in Asian countries. Endoscopic mucosal resection and endoscopic submucosal dissection have become the preferred therapeutic techniques in Japan and Korea for the treatment of early gastric cancer patients with a very low risk of lymph node metastasis. Laparoscopic and robotic resections for early gastric cancer, including function-preserving resections, have propagated through advances in technology and surgeon experience. The aim of this paper is to discuss the recent advances in minimally invasive approaches in the treatment of early gastric cancer.

Key words: Endoscopy; Endoscopic resection; Endoscopic mucosal resection; Endoscopic submucosal dissection; Laparoscopic resection; Early gastric cancer; Pylorus preserving gastrectomy; Sentinel lymph node; Robotic gastrectomy

Core tip: Early gastric cancer (EGC) is associated with favorable prognosis and there have been many efforts made to minimize the invasiveness of resection. Curative minimally invasive approaches utilized for EGC include endoscopic, laparoscopic and robotic approaches, and sentinel lymph node biopsy. Endoscopic resections have been shown to be safe and effective treatments for carefully selected patients with EGC. In patients with EGC that are not candidates for endoscopic resection, laparoscopic and robotic resections allow for the appropriate curative resection and lymphadenectomy with the benefits of minimally invasive surgery, including improved pain, reduced blood loss, and shorter hospital length of stay.

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INTRODUCTION

Although the incidence of gastric cancer has declined, it remains one of the most common causes of cancer-related mortality worldwide[1,2]. There are noted regional differences in gastric cancer epidemiology between East Asian and Western nations. In Japan and Korea, where the incidence of gastric cancer remains high, population-based screening with double-contrast barium radiography and/or endoscopy has allowed for earlier detection and presumably better survival[3,4]. Analysis of a Japanese nationwide

registry of gastric cancer revealed that 48.8% of cases currently treated are early stage disease[5]. However, in the West, late presentation of the disease still predominates[6].

Surgical resection remains the cornerstone of treatment in gastric cancer and prognosis is dependent on the stage at time of detection. Early gastric cancer (EGC) is defined as cancer in which tumor invasion is confined to the mucosa or submucosa (T1 cancer), regardless of lymph node involvement[7]. Long term survival data from Japan revealed that the 5-year cancer specific survival rates of EGC are 99% when limited to the mucosa and 96% when the submucosa is invaded[8,9]. Furthermore, depth of cancer invasion plays a role in the risk of lymph node (LN) metastasis. When gastric cancer is limited to the mucosa, the incidence of LN metastasis is less than 3% and rises to approximately 20% with submucosal involvement[8,9].

As EGC is associated with favorable prognosis, there have been many efforts made to minimize the invasiveness of resection. Minimally invasive approaches utilized for curative treatment of EGC include endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD), laparoscopic and robotic approaches, and sentinel lymph node biopsy[10]. The aim of this paper is to describe and discuss the recent advances in minimally invasive approaches in the treatment of EGC.

ROLE OF THERAPEUTIC ENDOSCOPY IN THE TREATMENT OF EARLY GASTRIC CANCER

Endoscopic resection techniques in the treatment of EGC

Endoscopic approaches in the treatment of EGC were first performed in Japan in 1974[8], but it was not until 1984 that EMR was first described[11]. Initially, EMR technique involved injecting saline under the lesion thus raising the tissue and allowing it to be grasped for snaring[11]. Over time, EMR has evolved through the use of different injection solutions, such as hypertonic saline with dilute epinephrine, addition of cap-fitted panendoscopes, and variceal ligation devices to capture the lesions[12-15]. The main disadvantage of EMR is that for lesions larger than 15mm, a piecemeal pathological specimen is inevitable, greatly impacting pathologists' ability to adequately stage patients[8,16]. ESD was developed at the National Cancer Center Hospital in Japan to overcome the limitations of EMR. In comparison with EMR, ESD allows for the resection of larger EGC lesions *en bloc* by dissection along the submucosal plane, thus preserving the specimen for more accurate pathologic assessment[17-20]. Resection with ESD, however, requires more advanced endoscopic skills and instrumentation to perform.

Pathological specimen processing

Endoscopic resection provides a specimen that will allow for assessment of the depth of tumor invasion, degree of differentiation and presence of lymphovascular invasion[21,22]. Assessment of the horizontal and vertical margins of the specimen are completed to confirm adequate resection[23]. Although, no lymph nodes are assessed pathologically, this information allows for prediction of the risk of LN metastasis based on published data of patients with similar pathological staging[24]. Importantly, both EMR and ESD allow for pathological staging without undermining any future surgical intervention.

Indications for endoscopic resection

EGC carries a favorable prognosis when treated with standard surgical resection and lymphadenectomy. Since EMR and ESD are not accompanied by lymphadenectomy, it is imperative to carefully determine the indications for endoscopic resection[25]. Ideally, endoscopic resection would be reserved for small, intramucosal EGC of intestinal histology type, in which LN involvement is very unlikely[8,25]. Large lesions, or those with diffuse histology type, are more likely to invade into the submucosa and exhibit metastasis to the LNs, making them poor candidates for endoscopic resection[26]. In Japan, indications for EMR and ESD are for well-differentiated EGC confined to the mucosa (depth T1a), measuring less than 2 cm in diameter, and without ulceration[23]. In the Unites States, National Comprehensive Cancer Network guidelines for tumors confined to the mucosa state that EMR is considered appropriate for lesions less than 1.5 cm, and ESD for lesions less than 3 cm[27]. Lesions selected for endoscopic resection should be devoid of lymphovascular invasion[28]. Importantly, these guidelines recommend that endoscopic resection for EGC be performed at high-volume centers.

The application of ESD has been explored beyond the standard indications for cancers with a very low probability of LN metastasis. Extended indications were proposed following the study of 5265 patients with EGC who underwent a gastrectomy and D2 lymphadenectomy by Gotoda *et al*[29], which revealed that these patients had no risk or a lower risk of lymph node metastasis than risks of mortality from a gastrectomy. Proposed extended indications for ESD include T1a tumors that are (1) differentiated without ulceration beyond 2 cm in size; (2) differentiated with ulceration up to 3 cm; and (3) undifferentiated without ulceration up to 2 cm. Large scale feasibility studies showed no differences in the 5-year overall (97.1%) and disease-specific (100%) survival rate of curative resection between the primary and expanded indications for endoscopic resections[30]. However, these extended indications remain investigational. Long-term ESD results from prospective clinical trials by the Japan Clinical Oncology Group (JCOG 0607 study) are pending, which may validate the expanded ESD indications[31]. JCOG 0607 study, a phase Ⅱ trial with 330 patients enrolled from 26 institutions, aims to evaluate the efficacy, safety and 5-year overall survival (OS) of patients undergoing ESD resection of T1a EGC under the expanded endoscopic treatment guidelines[31].

Outcomes for endoscopic resection

Although no randomized controlled studies (RCTs) exist comparing endoscopic resections with formal surgical resections[32], cohort studies have revealed that EMR treated patients had 5 and 10 year disease-specific survival of greater than 95% and the incidence of recurrence is approximately 6%[33]. In addition, these studies revealed that endoscopic approaches had favorable complication rates and quality of life compared to formal surgical resections[33]. ESD has also been shown to result in higher complete resection rates and recurrence-free rates when compared to EMR[34].

Complications from endoscopic resections include pain, bleeding and perforation. To prevent delayed bleeding following therapeutic endoscopy, patients are kept fasting the day of the surgery and are asked to begin fluid intake the day following resection and to resume a regular diet the second day after resection[8]. Resected gastric submucosal beds close within 6-8 wk, and patients are discharged on proton pump inhibitors for that duration[8,25]. Perforations are commonly closed with the aid of endoclips and often do not require additional surgical intervention[8,25]. Although Oda *et al*[34], in their retrospective multicenter study, revealed that the 3-year recurrence-free rate was higher with ESD than EMR (97.6% *vs* 92.5% respectively), ESD also proved to be associated with higher perforation rates (3.6% *vs* 1.2% respectively).

Follow-up after endoscopic resection

Endoscopic surveillance following definitive treatment of gastric cancer is required to monitor for evidence of recurrence. Abnormalities including mucosal surface changes, wall thickening or stricture, should be investigated with multiple biopsies (4-6) and alongside endoscopic ultrasound (EUS)[27]. Treatment of recurrence with further endoscopic resections is controversial.

ROLE OF LAPAROSCOPY IN THE TREATMENT OF EARLY GASTRIC CANCER

Laparoscopic resection techniques in the treatment of EGC

Although therapeutic endoscopy has become a standard treatment modality for selected EGC lesions, formal gastrectomy with lymphadenectomy remains the gold standard for most gastric cancers. Increasingly, laparoscopic resection has been used in the minimally invasive treatment for EGC[10,35]. Laparoscopic approaches that have been described for the treatment of EGC cancer include (1) Laparoscopic intragastric mucosal resections (LIGMR); (2) Laparoscopic wedge resection (LWR); and (3) Laparoscopic gastrectomy (LG).

Initially, laparoscopic resection techniques were used in the treatment of EGC that was strictly limited to the mucosa with no risk of lymph node involvement[36]. LIGMR, which was first described by Ohashi *et al*[37], involves the placement of 3 balloon trocars into the abdomen and into the lumen of the stomach through the anterior wall. The balloon equipped ports, one for the laparoscope and two for laparoscopic instruments, prevent air leak and fix the ports to the gastric wall[37]. LIGMR enabled mucosal resection of any part of the stomach except for the anterior wall while preserving the muscularis propria[37]. LWR, which allows for a full-thickness resection of lesions from the anterior stomach wall, was performed after endoscopic confirmation of an accessible lesion. Two approaches have been described. LWR can be performed using the "lesion lifting method", which entails introduction of a hollow needle at the point of the lesion for the application of a T-tack in the lumen of the stomach. The T-tack serves as an anchor lifting the lesion allowing it to be resected with a laparoscopic stapler[38]. The second method for LWR, first described by Kitano *et al*[39], involves making an incision in the seromuscular layer of the anterior stomach wall over the lesion, causing the mucosal lesion to bulge through and allowing for resection. The seromuscular layer is then sutured to close the defect[39]. As endoscopic techniques of EMR and ESD have become established as safe and effective treatment strategies for EGC confined to the mucosa, the use of LIGMR and LWR have largely decreased[36].

LG is increasingly used for the treatment of EGC with potential lymph node involvement[36]. In Japan and Korea, EGC is considered the only indication for laparoscopic gastrectomy. Several RCTs have been published comparing laparoscopic to open gastric resection conducted mainly in patients with EGC[40-43]. These mostly single-center studies have favorably supported laparoscopic resection for EGC, with benefits including reduced operative blood loss, less post-operative pain and earlier discharge from hospital[44-46]. A recent meta-analysis has found that patients undergoing LG were associated with faster return of bowel function but longer operative times and less harvested lymph nodes[47]. Ongoing RCTs are being performed to determine whether there is a significant difference in oncologic outcomes between the two groups. The Japan Clinical Oncology Group (JCOG 0912 study) and the Korean Laparoscopic Gastrointestinal Surgery Study Group (KLASS 01 Study) have initiated large multi-center RCTs comparing long-term survival for EGC following laparoscopic gastrectomy and open gastrectomy[48,49].

In addition, in Korea, a separate phase Ⅲ study (KLASS 02) has been initiated to evaluate the feasibility of laparoscopic resection in advanced gastric cancer (AGC) patients[48]. As we await those results, a recent systematic review and meta-analysis comparing LG with OG for AGC, performed by Chen *et al*[50], revealed similar safety and oncologic outcomes to those seen in the treatment of EGC. In the treatment of AGC, studies consistently revealed a reduction in intra-operative blood loss during LG in comparison to OG[50]. Although delicate dissection along with the complexity of performing

an adequate lymphadenectomy during LG was shown to be more time consuming and requiring extensive technical expertise[50], studies have shown a learning curve of approximately 50 LG cases before operative times can be reduced[51-53] and that times were not longer for LGs performed in large high-volume specialized centers[54,55]. As shown in studies evaluating LG for EGC, Chen *et al*[50] also revealed a reduced number of post-operative complications (*i.e.*, wound infections, respiratory complications), reduced use of analgesic use, and earlier return of bowel function in the LG group for AGC. Furthermore, their systematic review revealed that LG for AGC had similar cancer recurrence and long-term survival rate to patients treated by OG[50]. Therefore existing studies show that LG for the treatment of AGC is both safe and feasible, and results from large multi-center RCTs with extended follow up will shed more light on its oncologic applicability[50].

Combination of endoscopic and laparoscopic approaches

Laparoscopic and endoscopic cooperative surgery (LECS) was developed by Hiki *et al*[56] and Nunobe *et al*[57] for the dissection of submucosal tumors of the stomach. The LECS technique involves initial endoscopic identification and confirmation of tumor location followed by ESD[56,57]. Laparoscopic serosal dissection is performed and a stapling device is applied to close the incision line[56,57]. LECS is indicated in the treatment of EGCs larger than 3 cm in diameter located at the greater curvature of the stomach or for lesions with extensive ulcerations that may not be amenable to ESD[57]. Importantly, LECS does not involve lymphadenectomy.

Combining endoscopic resection with laparoscopic lymphadenectomy has also been investigated in cases where lymph node involvement cannot be disregarded[58,59]. Abe *et al*[59] noted early and delayed gastric ischemia of the preserved stomach secondary to division of major feeding arteries during lymphadenectomy, which resulted in gastric perforation in 1 of 21 patients. In addition, 2 out of 21 patients exhibited gastric emptying problems, although preoperative quality of life was maintained with no dietary restrictions[59]. Further studies are necessary before this becomes an acceptable alternative to gastrectomy without compromising oncologic principles[59].

ROLE OF FUNCTION-PRESERVING RESECTIONS AND LAPAROSCOPY

Resection techniques have been developed with the aim of reducing the functional sequelae of radical gastric resections including dumping syndrome, reflux gastroesophagitis and weight loss[60]. Minimally-invasive procedures combining laparoscopic resections with function-preserving gastric surgery include (1) pylorus-preserving gastrectomy (PPG) for distal lesions; (2) proximal gastrectomy (PG) for proximal lesions; and (3) laparoscopic subtotal with small remnant gastric pouch for proximal lesions.

Laparoscopic pylorus-preserving gastrectomy

Pylorus-preserving gastrectomy (PPG), which was originally limited to the treatment of benign gastric diseases such as gastric ulcers[61], has become an increasingly accepted treatment modality for EGC patients. The preservation of pyloric function in gastric resections has shown improvements over conventional distal gastrectomy in the prevention of dumping syndrome[62], the prevention of bile reflux[63] and reduced post-operative weight loss[64]. Laparoscopic-assisted PPG (LAPPG), which introduces the benefits of laparoscopic surgery, including lower post-operative pain, shorter hospitalization, early return of bowel function, and better cosmesis, is a modality for the treatment of EGC in many institutions in Japan and South Korea[65]. LAPPG involves preservation of the right gastric artery and the pyloric branch of the vagus nerve required to maintain pyloric circulation and motility[64,66]. However, there are concerns that LAPPG does not allow for adequate suprapyloric lymph node dissection[67]. Studies that have evaluated the incidence of lymph node metastasis following distal gastrectomies for EGC have found a 4% rate of metastasis to the suprapyloric lymph nodes[68,69], although 29%-34% of those patients were found to be T2-T3 gastric cancer after final pathological evaluation[67]. A retrospective survey of the Gastric Cancer Data Base in Japan by Akiyama *et al*[12] revealed a 0.2% metastasis rate of the suprapyloric lymph nodes after evaluation of 3646 cases of T1 tumors located in the body of the stomach.

Indications for performing LAPPG include (1) intramucosal or submucosal gastric adenocarcinoma without lymph node involvement (cT1, cN0); and (2) tumor lesion located in the distal stomach (4.5 cm to 5 cm proximal to the pyloric ring)[65]. A study performed by Morita *et al*[70], evaluating 611 patients who underwent a PPG for T1 gastric cancer had a 5-year OS rate of 96.3%. Hiki *et al*[71] evaluated 305 patients treated by PPG and revealed a 5-year OS rate of 98%. While Jiang *et al*[72] evaluated 188 patients who underwent a LAPPG and revealed a 3-year OS rate and 3-year disease-specific survival rate of 97.8% and 99.3%.

Laparoscopic proximal gastrectomy and laparoscopic subtotal gastrectomy with small remnant pouch

Proximal tumors are commonly treated with a total gastrectomy[73]. Laparoscopy-assisted total gastrectomy (LATG) is a technically difficult procedure relative to a laparoscopy-assisted distal gastrectomy (LADG) and is associated with higher rates of post-operative complications of increased operative blood loss and increased length of hospitalization[74,75]. In addition, Lee *et al*[76] showed that LATG was associated with an increased rate of anastomotic stricture in comparison to LADG (26.9% *vs* 8.0%, respectively).

PG has been proposed as a function-preserving approach for EGC[67]. Due to the association with mark-

edly higher rates of complications including anastomotic stenosis, reflux esophagitis and no change in nutritional status in comparison to total gastrectomies, An *et al*[77] concluded that PG are not a better option than total gastrectomy for proximal third EGC. There has been no apparent advantage with laparoscopic-assisted PG (LAPG)[78].

To improve post-operative quality of life, Jiang *et al*[79] have developed a novel approach for selected patient with proximal EGC, laparoscopy-assisted subtotal gastrectomy (LAsTG), which involves preserving a small proximal gastric pouch. LAsTG carries some concerns pertaining to oncological and reconstruction safety with the preservation of a limited remnant stomach[67]. The indications for LAsTG include (1) a pre-operative diagnosis of T1N0 EGC; (2) tumor location is in the proximal third of the stomach; (3) distance between tumor and gastroesophageal junction (GEJ) of 5 cm; and (4) remnant gastric stump measuring 2-3 cm from GEJ[67].

ROLE OF ROBOTIC ASSISTED GASTRECTOMY IN THE TREATMENT OF EARLY GASTRIC CANCER

Robot-assisted gastrectomy (RAG) may allow surgeons to overcome some of the technical limitations of laparoscopic resections for EGC[80]. Robotics improves visualization by providing a magnified, high-definition, three-dimensional image that allows the surgeon to identify smaller anatomical structures[81]. In addition, manipulation of tissue is improved with the elimination of physiologic tremor and articulating tools providing seven degrees of freedom and reproducing the movement of the human hand[81]. Accordingly, RAG may be advantageous to performing the more technically challenging D2 lymphadenectomy[81]. Articulating robotic instruments may allow for the dissection of LNs from difficult lymphatic stations around major vessels and organs[81].

Long-term survival results following RAG are required to assess oncological outcomes, however studies have shown this approach to be adequate in terms of resection margins, lymphadenectomy and staging[82,83]. No differences were noted in terms of the number of lymph nodes harvested when comparing open, laparoscopic and robotic gastrectomy, however the estimated blood loss was significantly less in the robotic group in comparison with the other two approaches[84]. A recent meta-analysis of three non-randomized controlled trials was performed by Xiong *et al*[85]. Operative time was significantly longer in the RAG group in comparison to the LG group but was associated with significantly less intra-operative blood loss[85]. Furthermore, the comparison of RAG with LG revealed no differences in the number of lymph nodes harvested, length of hospitalization, and morbidity and mortality rates[85]. In addition, several studies have reported shorter learning curves for RAG compared to LG (20 cases *vs* 50 cases, respectively). Further studies are required to assess oncological outcomes following RAG, as well as addressing important concerns regarding cost-effectiveness[81].

ROLE OF SENTINEL LYMPH NODE BIOPSY IN THE TREATMENT OF EARLY GASTRIC CANCER

Accurate assessment of lymph node status is an integral part to determination of clinical outcomes and for therapeutic planning in gastric cancer. EGC is associated with 5-year OS rates of greater than 90% and pathological data have suggested that the majority of lymph nodes resected do not contain metastases[29,86-88]. Further, extensive lymphadenectomies are associated with increased risk of complications[89]. Sentinel lymph node (SLN) biopsy is well-established in the treatment of breast cancer and melanoma, and allows for lymph node assessment with limited dissection and reduced complications[90]. SLN biopsy has been investigated as an alternative to extensive lymphadenectomy in the treatment of EGC. Mapping for SLN biopsy has been completed with dye, radio-colloid, as well as combinations of dye and radio-colloid. Potential anatomical limitations to SLN mapping exist in gastric cancer, due to the complex and unpredictable lymphatic drainage of the stomach, increasing the likelihood of skip metastases.

A systematic review on the accuracy of SLN biopsy in gastric cancer was performed by Cardoso *et al*[91]. This study revealed an overall calculated false negative rate (FNR) of 34.7% with dye alone, 18.5% with radio-colloid alone, and 13.1% for the combination of dye and radio-colloid[91]. A recent systematic review performed by Can *et al*[92], reveals accuracy rates ranged from 78% to 100%. In addition, there has been publication of the results of a multicenter trial (JCOG study 0302), which evaluated the feasibility and accuracy of diagnosis using SLN biopsy in T1 gastric cancer[93]. Final results revealed a high FNR and accrual was suspended early. Primary analysis revealed a FNR of 46% (13/28) and 7 of 13 patients had nodal metastases outside the lymphatic basin[93]. However, a recent prospective multicenter trial in Japan performed by Kitagawa *et al*[94], revealed a higher accuracy of nodal evaluation for metastasis (93%) and lower FNR (7%) compared to JCOG 0302 results. This drastic difference in results may be explained by the difference in the procedural learning phase in both studies[94]. In JCOG 0302, only five cases were required as the minimum for the initial leaning phase, while a minimum of 30 cases were required for the learning phase in the multicenter trial performed by Kitagawa *et al*[94]. Thus at present, SLN biopsy remains an experimental treatment modality in gastric cancer[93].

FUTURE DIRECTIONS

Novel surgical approaches, including natural orifice

transluminal endoscopic surgery (NOTES) and single-incision laparoscopic surgery (SILS), are currently being investigated as minimally-invasive treatment options for EGC[95]. NOTES entails incision-less surgery to access the peritoneal cavity through natural orifices[96]. Although it has been applied sporadically in bariatric surgery, Nakajima *et al*[97] have shown that transvaginal NOTES may represent an option for performing partial gastrectomy for patients with gastric submucosal tumors. Hybrid procedures are being developed including NOTES with SLN biopsy and NOTES with laparoscopy with the goal of expanding indications for its application[96,98]. In comparison to NOTES, which is still in early stages of development, SILS shows earlier promise in the treatment of gastric cancer. SILS is frequently applied in appendix, gallbladder, colon and bariatric surgery[95]. With favorable cosmetic results, Omori *et al*[99] demonstrated SILS distal gastrectomy as a feasible and safe approach for EGC, while Ahn *et al*[95] performed the first SILS total gastrectomy with D1 lymphadenectomy for proximal EGC. As instrumentation improves and surgeon experience increases, these novel approaches show potential in improving cosmesis and reducing post-operative pain in comparison to the current laparoscopic approaches.

CONCLUSION

The prognosis of gastric cancer patients can be improved by early detection and treatment. Minimally-invasive approaches to patients with early gastric cancer have been developed to improve quality of life without compromising oncologic outcomes. EMR and ESD have been shown to be safe and effective treatments for carefully selected patients with EGC. Long term clinical trial results are still pending from Japan for extended criteria, and it is likely that endoscopic approaches have an increasing role in the treatment of EGC. In patients with EGC that are not candidates for endoscopic resection, laparoscopic and robotic resections allow for the appropriate curative resection and lymphadenectomy with the benefits of minimally invasive surgery, including improved pain, reduced blood loss, and shorter hospital length of stay. Growing interest in minimally invasive function-preserving resections will need to be supported with further study to assess oncologic safety. The roles of laparoscopy combined with endoscopic resections as well as SLN biopsy remains to be determined. Important to all these advancements in the treatment of EGC is the continued efforts to assess safety and function without compromising curability.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Extent of lymphadenectomy and perioperative therapies: Two open issues in gastric cancer

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Abstract

Gastric cancer is one of the leading causes of death for cancer worldwide, although geographical variations in incidence exist. Over the last decades, its incidence and mortality have gradually decreased in Western countries, while these have increased, or remained stable, in the other world regions. Gastric cancer is often diagnosed at an advanced stage, with the only notable exception of Japan, where nationwide screening programs are enforced, due to local high incidence. Curative-intent surgery (*i.e.*, gastrectomy, total or partial, and lymphadenectomy) remains the cornerstone of treatment of gastric cancer. Much has been debated about the extent of lymph node dissection and, although it is a valuable contribution to staging and cure, operative treatment only represents one aspect of overall effective management, as the risk of both locoregional and distant recurrences are high, and bear a poor prognosis. As a matter of fact, surgery, as a single modality treatment, has probably achieved its maximum efficacy for local control and survival, while other accompanying nonsurgical treatment modalities have to be taken into account, although their role is still the subject of considerable debate. The authors in this review present an update on the outcome of treatment of gastric cancer in relation to the extent of lymphadenectomy and of various nonsurgical preoperative, intraoperative, and postoperative strategies.

Key words: Gastric cancer; Adenocarcinoma; Postoperative; Preoperative; Chemoradiotherapy; Chemotherapy; Radiotherapy; Intraperitoneal; Randomized controlled trial; Meta-analysis

Core tip: The authors in this review present an update on treatment of gastric cancer in relation to the role of extent of lymphadenectomy and of new nonoperative strategies, to employ preoperatively, intraoperatively, and postoperatively. The above therapeutical options are assessed by reviewing the most authoritative, large, and referenced randomised controlled trials and meta-analyses published in the English literature.

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INTRODUCTION

Gastric cancer is one of the most common malignancies in the world. Over the last decades, its incidence and mortality have gradually decreased in Western countries, while these have increased, or remained stable, in the other world regions. In countries with a higher incidence, nationwide endoscopic screening programs lead to earlier identification of a large number of gastric cancers, while,

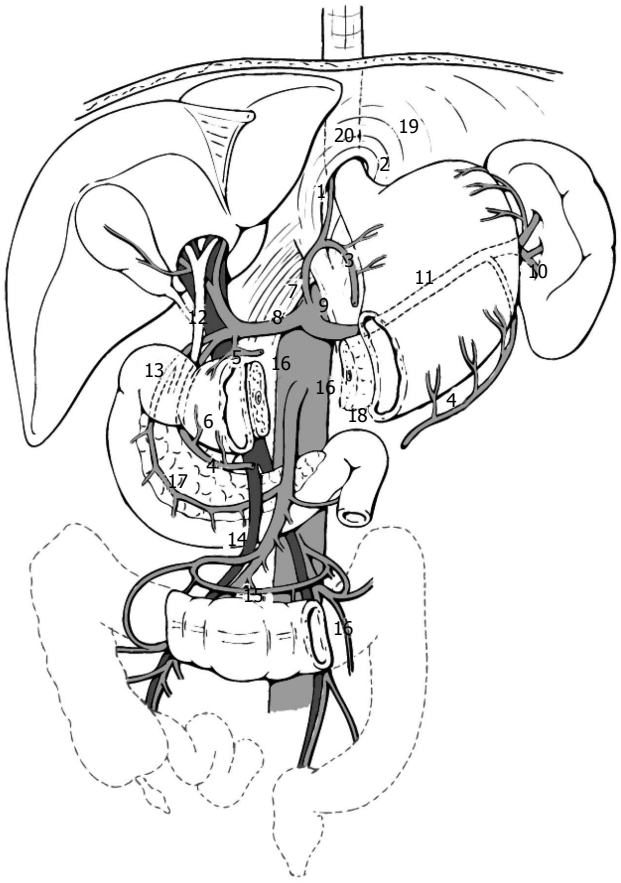

Figure 1 Location of gastric lymph node stations according to Japanese Research Society for Gastric Cancer (JRSSC)[10]. For description of numbers, see Table 1.

Table 1 Regional lymph nodes as defined by the Japanese Research Society for Gastric Cancer (JGCA, 1998)[10]

No. 1 Right paracardial LN
No. 2 Left paracardial LN
No. 3 LN along the lesser curvature
No. 4 LN along greater curve
(short gastric vessels, left and right gastroepiploic vessels)
No. 5 Suprapyloric LN
No. 6 Infrapyloric LN
No. 7 LN along the left gastric artery
No. 8 LN along the common hepatic artery
(anterosuperior and posterior group)
No. 9 LN around the celiac artery
No. 10 LN at the splenic hilum
No. 11 LN along the splenic artery (proximal and distal tract)
No. 12 LN in the hepatoduodenal ligament (along hepatic artery, bile duct and portal vein)
No. 13 LN retropancreatic
No. 14 LN along superior mesenteric vessels (vein and artery)
No. 15 LN along the middle colic vessels
No. 16 LN paraaortic (of upper, middle and lower abdominal aorta, in relation to the intragastric tumor site)
The classification includes also the following lymph node compartments:
No. 17 LN on the anterior surface of the pancreatic head
No. 18 LN along the inferior margin of the pancreas
No. 19 Infradiaphragmatic LN[1]
No. 20 LN in the esophageal hiatus of the diaphragm[1]
No. 110 Paraesophageal LN in the lower thorax[1]
No. 111 Supradiaphragmatic LN[1]
No. 112 Posterior mediastinal LN[1]

[1]When the gastric carcinoma also invades the esophagus. LN: Lymph nodes.

in Western countries, the lack of similar screening programs often causes later diagnoses.

Surgery with lymphadenectomy is the best treatment option for resectable gastric cancer, as it provides better results both in terms of progression-free survival and prognosis. Long-term survival after radical surgery strongly depends on the stage of the tumor. The resection of tumors limited to the mucosa shows a survival rate of 90%-95%[1-3]. On the other hand, progression of the disease through the gastric wall and/or through regional lymph nodes shows higher recurrence rates of the disease, with five-year survival rates lower than 30% in Western countries[3]. Over the last decades, this has led to the development of further treatment options, such as extension of lymphadenectomy, and the use of pre-, intra-, and postoperative chemoradiotherapy. Indication, timing, and effectiveness of these options, however, are still controversial. The aim of this paper is to report on the current views on treatment of gastric cancer, and to possibly clarify the topics introduced above. Data from randomized controlled trials (RCT) and meta-analysis are reported. RCTs are considered the gold standard of all research design, while meta-analysis provide a comprehensive up-to-date summary of the average effect of all the relevant RCTs and are a reliable guidance for clinical practice and future research.

PROBLEM OF LYMPH NODE DISSECTION

Gastric carcinoma shows a high tendency to lymph node metastasis. The risk of regional nodal involvement increases with deep penetration through the gastric wall[4], and the nodal extension of the cancer takes place gradually, radiating from primary location *via* the lymphatic system[5,6]. Nodal metastases are observed in 3%-5% of gastric carcinomas which are limited to the mucosa, in 11%-25% of those which extend to the submucosa, in 50% of those which reach the muscularis (T2), and in 83% of those which extend to the serosa (T3)[7,8]. After curative radical resection, local recurrence is represented, in 87.5% of cases, by nodal metastases to local or regional lymph node stations[9]. The Japanese Classification of Gastric Carcinoma (Japanese Gastric Cancer Association, JGCA, 1998)[10] has defined 16 different lymph node stations (n) which drain the stomach. These are subdivided in three levels, according to their distance from the tumor (Figure 1, Table 1), thus entailing three types of lymph node dissection (D) that can be associated to total or partial gastrectomy: D1, in which perigastric lymph nodes from n1 to n6 are removed (N1 level); D2, in which perigastric lymph nodes are removed as well as those located along the main arterial vessels from n7 to n12 (N2 level); D3, in which stations n13 to n16 are removed, as well as those mentioned before (N3 level) (Table 2). During the '60s, the Japanese authors first introduced D2 lymphade-

Table 2 Nodal compartments to be removed for each type of lymph node dissection as defined by the Japanese Research Society for Gastric Cancer (JGCA, 1998)[10]

Tumor site	LN D1 dissection	LN D2 dissection	LN D3 dissection
Upper stomach	1, 2, 3, 4	4, 7, 8, 9, 10, 11	5, 6, 8, 12, 16
Middle stomach	1, 3, 4, 5, 6	7, 8, 9, 11, 12	4, 8, 10, 11, 12, 13, 14, 16
Lower stomach	3, 4, 5, 6	1, 7, 8, 9, 11, 12, 14	4, 8, 12, 13, 16

LN: Lymph nodes.

nectomy[11], which they still consider as the standard procedure to associate with curative gastric resection, as it yields the best results in terms of local recurrence and of long term survival. In Western countries, D2 lymph node dissection is not as common as Japan, not only due to lower incidence of gastric cancer (and, consequently, to the fact that surgeons have less experience with this technique), but mainly to high morbidity and mortality linked to this type of lymph node dissection. Indeed, D2-D3 lymphadenectomy is a challenging surgical procedure, which implies a thorough surgical training conducted under the supervision of experienced gastric surgeons[12]. A significant difference between Japanese clinical outcomes and those of other countries has been observed in short- and long-term results and in loco-regional control, with better results for Japanese clinical records. The systematic lymph node dissection practiced since the '60s-'70s by Japanese surgeons may have contributed to achieve better results[13]. The International Union Against Cancer (UICC)[14] adopted in 1997 a new classification system for lymph node metastases, which, unlike the Japanese system, was not based on the anatomic location of positive nodes[15], but on their number. It was recommended that at least 15 lymph nodes should be removed and examined for proper staging. Secondaries affecting 1 to 6 lymph nodes were classified as pN1, from 7 to 15 as pN2, more than 15 as pN3. It has been shown[16] that lymph node removal was much higher with D2 resection (more than 25 lymph nodes) than with D1 (less than 25 lymph nodes), and that survival is related to the number of lymph nodes with metastasis[17-19]. The extent of lymphadenectomy, therefore, has always been controversial. Most Western surgeons criticize D2 dissection because some benefits have only been confirmed in retrospective observations[20]. RCTs, mainly conducted by Western authors, since the '80s compare short-term and long-term results in D1 and D2 resections. Dent performed a RCT on 43 cases[21], and the group who received D2 resection (21 patients), showed worse results compared to the D1 lymphadenectomy group (22 patients) in terms of duration of surgery, blood transfusion requirement, postoperative morbidity, and duration of hospital stay. Four patients in D2 group required reoperation; in both groups there were no postoperative deaths. There was no difference in the probability of survival at a median follow-up of 3 years. In the mid-'90s, the Dutch Gastric Cancer Trial[22] and the Medical Research Council (MRC) Gastric Cancer Surgical Trial (STO1)[23] published the morbidity and mortality results of two multicentric RCTs. The studies, conducted, respectively, on 711 and 400 patients receiving curative D1 or D2 resection for advanced gastric carcinoma, showed a higher incidence of postoperative complications and mortality and longer duration of hospitalization in patients treated with more extended lymph node dissection. Both studies also showed that the higher number of postoperative complications and mortality observed in the D2 group were not linked to the extent of the lymphadenectomy as such, rather to associated pancreatectomy and/or splenectomy. The protocol of D2 lymph node dissection, in case of total gastrectomy, included these resections[10,24] in order to remove all lymph nodes in stations 10 and 11 (Table 1). In the late '90s[25,26], the same authors reported the long-term survival data. Both trials showed that, 5 years after surgery, the survival rate, risk of relapse, risk of death for cancer and duration of disease free survival were not significantly different in D1 and D2 groups. However, in the MRC STO1 trial[26], a better long-term survival was observed in patients receiving D2 gastrectomy without pancreatectomy and/or splenectomy. On the basis of these data, the British National Health Service Cancer Guidance in 2001 discouraged the use of D2 resection in routine clinical practice[27]. On the other hand, lymphadenectomies even more extended than D2 have been performed since the '80s in several specialized Japanese centers, on the grounds that lymph node para-aortic metastases (N3) were frequently observed (20%-30% of cases)[28,29]. Some Japanese authors (JCOG 9501)[30] published in 2004 a multicentric RCT on 523 patients receiving surgical treatment for gastric cancer, comparing short-term results in D2 standard and in D2 extended to para-aortic lymph nodes. In the extended para-aortic lymphadenectomy group, duration of surgery and intraoperative blood loss were significantly higher (P = 0.0001), while mortality and reoperation rates were not statistically different compared to standard D2 group. However, morbidity in the more extended surgery group was slightly higher than in the standard group (P = 0.067). Uni- and multivariate analysis later conducted by the same authors[31], showed that key factors for complications were: age > 56 (P = 0.026), associated pancreatectomy (P = 0.004), duration of surgery > 297 min (P = 0.045) and a body mass index (BMI) ⩾ 25 (P = 0.002). The long-term results of the same trial[32] were published in 2008, showing no significant differences between standard and extended para-aortic lymphadenectomy in terms both of 5-year overall survival (69.2% *vs* 70.3%) and of 5-year disease-free survival (62.6% *vs* 61.7%). Interestingly, extended D2 resection only showed better results in terms of 5-year survival in patients without lymph node metastasis (HR for death 0.39; P = 0.009), while it resulted pointless in those with lymph node metastases (HR for death 1.39; P = 0.04). Based on these data, standard D2 resection was judged as adequate by the authors for

patients with potentially curable advanced gastric carcinoma. Short-[33] and long-term[34] results of a comparative RCT between D1 and D3 (the D3 definition reported in[34] did not include para-aortic lymph nodes), conducted on 221 patients who received curative surgery in a single-institution (Taipei Veterans General Hospital, Taiwan) were reported in 2004 and 2006. No cases of operative mortality were observed in the two groups. Duration of surgery, blood loss, blood transfusion required, volume of abdominal drainage, duration of postoperative stay ($P = 0.001$), and number of surgical complications ($P < 0.001$) resulted higher in D3 group. The incidence of complications was higher ($P = 0.017$) in patients receiving resection with distal pancreatectomy and splenectomy. Overall 5-year survival was higher in D3 group (59.5% *vs* 53.6%, $P = 0.041$), while 5-year recurrences after R0 (radical resection) showed no difference (50.6% in D1 group and 40.3% in D3 group, $P = 0.197$). The authors concluded that D3 dissection improves survival rates, and suggested that it should be performed in specialized centers in order to limit the chance of postoperative complications. A RCT conducted by the East Asia Surgical Oncology Group in 2008[35] compared data of 135 patients treated with D2 gastrectomy with those of 134 patients receiving D4 gastrectomy (in D4 dissection inter-, pre-, and latero-aortic lymph nodes of abdominal aorta as far as bifurcation are removed). No significant advantages were observed in terms of 5-year survival in patients who received extended lymphadenectomy ($P = 0.80$). Twelve patients of D4 group with metastases to para-aortic lymph-nodes had a median survival of 2.8 years, and a 5-year survival rate of 25%. The authors maintained that D4 dissection is not the best treatment option for patients with gastric carcinoma, whereas D2 dissection is recommended if performed by experienced surgeons. The Dutch Gastric Cancer Group Trial[36], published in 2004, updated data on survival of 711 patients previously enrolled in published RCTs[22,25]. At a median follow-up of 11 years, survival rates were 30% in D1 group and 35% in D2 group ($P = 0.53$), the risk of recurrence was 70% and 65%, respectively ($P = 0.43$). The authors concluded that D2 lymph node dissection can be recommended only if operative morbidity and mortality can be reduced. A further update of these data was published in 2010[37], with a median follow-up of 15.2 years. The overall 15-year survival was 21% after D1 resection and 29% after D2 resection ($P = 0.34$). Gastric cancer-related mortality rates resulted significantly higher in D1 than in D2 (41% *vs* 37%; $P = 0.01$). The incidence of local recurrence (D1 = 22% *vs* D2 = 12%) and distant recurrence (D1 = 19% *vs* D2 = 13%) were different, albeit not significantly. Patients who received splenectomy and pancreatectomy had significantly lower overall survival rates in both D2 and D1 groups. On the other hand, patients who received D2 resection without pancreatico-splenectomy had a significantly higher overall 15-year survival compared to patients receiving D1 resection (35% *vs* 22%, $P = 0.006$). The authors concluded that D2 resection should be considered the standard procedure to treat resectable gastric carcinoma. The Italian Gastric Cancer Study Group[38] in 2010 published a multicentric RCT on 267 patients, comparing the short-term results of D1 and D2 gastrectomy for curable gastric cancer. Pancreatico-splenectomy was not considered as a routine part of the D2 gastrectomy and spleen and pancreas were removed only when indicated by the surgeon. The study did not show significant differences in terms of operative mortality, morbidity and duration of postoperative hospital stay. The authors concluded that D2 gastrectomy is a safe option to treat gastric carcinoma of Western patients as well, if it is performed in specialized centers. Three meta-analyses of RCTs evaluating D1 *vs* D2 *vs* D3 lymphadenectomy for operable gastric carcinoma were conducted in 2009[39], 2011[40], and 2012[41]. These three studies examined 14, 6 and 5 RCTs, totaling 3432, 1876 and 1642 patients, respectively. The 2009 meta-analysis[39], conducted on Western and Asiatic trials, compared D1 and D2 dissections and D2 and D3 dissections. In the first comparison, duration of surgery, operative mortality and postoperative complications resulted significantly lower in D1 dissection than in D2 ($P = 0.00001$, $P < 0.001$, $P < 0.001$), while 3- and 5-year survival rates did not show significant differences. No substantial differences were found between D2 and D3 dissections in relation to operative mortality, postoperative morbidity, operative time, and hospital stay. The meta-analysis of 2011[40] also showed better results for D1 dissections, with shorter duration of postoperative hospitalization ($P = 0.0036$), lower incidence of mortality ($P = 0.0054$), complications ($P = 0.0002$), anastomotic dehiscence ($P = 0.0001$), and re-operations ($P = 0.006$). However, no differences were observed in 5-year survival ($P = 0.76$). The meta-analysis of 2012[41] confirmed a higher incidence of mortality and reoperations after D2 resection compared to D1 ($P = 0.02$ and $P < 0.0001$ respectively). The hospital mortality was significantly higher for D2 resections performed before 1995 ($P = 0.0003$), while after that date hospital mortality was no longer different between groups ($P = 0.70$). A further analysis showed that the difference in hospital mortality was related to associated distal pancreas and/or spleen removal. Patients in D2 group with spleen preservation had significantly lower hospital mortality than those who had their spleen resected ($P < 0.0001$). Overall 5-year survival was not significantly different in the two types of lymph node dissection ($P = 0.58$). Main data regarding the extent of nodal dissection are shown in Table 3.

In conclusion, in Western countries the prognostic value of D2 lymphadenectomy is still controversial, while in Eastern countries it is considered a standard procedure, likely to be further extended. Japanese authors do not even conduct RCT comparing D1 and D2 lymphadenectomies, on the grounds that they consider D1 dissection unethical. Data indicate that D2 dissection is an adequate and potentially beneficial staging and treatment approach if operative mortality is avoided. Dissections extended

Table 3 Main data regarding the extent of nodal dissection

Ref.	Study design	No. of patients	Post-operative mortality (*P* value)	Post-operative morbidity (*P* value)	Survival (*P* value)	Recurrence (*P* value)
Dent *et al*[21], 1988	RCT D1 *vs* D2	D1: 22; D2: 21	None	D1 < D2 (nv)	At 3 yr (NS)	-
Bonenkamp *et al*[22], 1995 Dutch D1D2 trial	RCT D1 *vs* D2	D1: 380; D2: 331	D1 < D2 (0.004)	D1 < D2 (0.001)	-	-
Cuschieri *et al*[23], 1996 MRC ST01	RCT D1 *vs* D2	D1: 200; D2: 200	D1 < D2 (0.04)	D1 < D2 (0.001)	-	-
Bonenkamp *et al*[25], 1999 Dutch D1D2 trial	RCT D1 *vs* D2	D1: 380; D2: 331	-	-	At 5 yr D1 *vs* D2 (NS)	At 5 yr D1 *vs* D2 (NS)
Cuschieri *et al*[26], 1999 MRC ST01	RCT D1 *vs* D2	D1: 200; D2: 200	-	-	At 5 yr D1 *vs* D2 (NS)	At 5 yr D1 *vs* D2 (NS)
Sano *et al*[30], 2004 JCOG Study 9501	RCT D2 *vs* D3	D2: 263; D3: 260	D2 *vs* D3 (NS)	D2 < D3 (NS)	-	-
Sasako *et al*[32], 2008 JCOG Study 9501	RCT D2 *vs* D3	D2: 263; D3: 260	-	-	At 5 yr D2 *vs* D3 (NS)	At 5 yr D2 *vs* D3 (NS)
Wu *et al*[33], 2004	RCT D1 *vs* D3	D1: 110; D3: 111	None	D1 < D3 (0.012)	-	-
Wu *et al*[34], 2006	RCT D1 *vs* D3	D1: 110; D3: 111	-	-	At 5 yr D1 < D3 (0.041)	At 5 yr D1 *vs* D2 (NS)
Hartgrink *et al*[36], 2004 Dutch D1D2 trial	RCT D1 *vs* D2	D1: 380; D2: 331	-	-	At 11 yr D1 *vs* D2 (NS)	At 11 yr D1 *vs* D2 (NS)
Songun *et al*[37], 2010 Dutch D1D2 trial	RCT D1 *vs* D2	D1: 380; D2: 331	-	-	At 15 yr D1 *vs* D2 (NS)	At 15 yr D1 > D2 (0.005)
Degiuli *et al*[38], 2010 IGCSG	RCT D1 *vs* D2	D1: 133; D2: 134	D1 *vs* D2 (NS)	D1 *vs* D2 (NS)	-	-
Yang *et al*[39], 2009	Meta-analysis D1 *vs* D2	D1: 907; D2: 875	D1 < D2 (0.001)	D1 < D2 (0.0001)	At 3 and 5 yr D1 *vs* D2 (NS)	-
	Meta-analysis D2 *vs* D3	D2: 599; D3: 588	D2 *vs* D3 (NS)	D2 *vs* D3 (NS)	-	-
Memon *et al*[40], 2011	Meta-analysis D1 *vs* D2	D1: 946; D2: 930	D1 < D2 (0.005)	D1 < D2 (0.0002)	At 5 yr D1 *vs* D2 (NS)	-
Seevaratnam *et al*[41], 2012	Meta-analysis D1 *vs* D2	D1: 845; D2 797	D1 < D2 (0.002)	D1 < D2 (0.0001)	At 5 yr D1 *vs* D2 (NS)	-

RCT: Randomized controlled trial; NS: Not significant; nv: Not valued.

to para-aortic lymph nodes does not show significant advantages in terms of survival. Splenectomy and distal pancreatectomy increase operative morbidity and mortality. D2 dissection is considered a difficult procedure, and should be performed by experienced surgeons in specialized centers. Authors suggest that a surgeon should perform at least 200 gastrectomies under the supervision of an experienced surgeon before he can perform D2 lymph node dissections with acceptable morbidity and mortality rates[12]. In Western countries, due to the lower incidence of gastric carcinoma, a surgeon is very unlikely to achieve such an experience.

PERIOPERATIVE THERAPIES

In Western countries, the 5-year survival rates for advanced gastric carcinoma treated with potentially curative surgery range between 25% and 30%[16]. Recurrences occur in the abdomen in 40%-60% of the cases, both as the only site and as part of a systemic diffusion of disease[9,42,43]. The most frequent abdominal sites of recurrence are the area previously occupied by the tumor, the anastomosis and the non-resected regional lymph nodes. These data show that surgery, as a single modality treatment, cannot detect and remove the satellite micrometastases around the primary tumor, nor the tumor cells disseminated during the operative maneuvers. Pre-, intra- and post-operative treatments have been developed in the last decades, in order to improve loco-regional control of disease and long term survival.

Adjuvant treatments

Adjuvant chemoradiotherapy: Adjuvant treatments for gastric carcinoma have been employed since the '70s[44-46], on the assumption that if surgery alone could not cure the disease, as shown by the high incidence of local and distant recurrences, adjuvant treatments could improve the outcomes by acting on the remaining tumor. Theoretically, adjuvant treatments should eradicate cancer cells already metastasized prior to surgery or accidentally disseminated during surgery. Therefore, the level of surgical radicality or the residual tumor after surgery can affect the results of adjuvant therapies. Furthermore, these treatments do not show significant benefits compared to surgery alone for early stage tumors (T1, N0)[10], in which surgery is likely to achieve the cure[47]. The results of one of the first RCTs on this issue were published in 1984[44]. The study was conducted on 62 patients receiving resective surgery for poor-prognosis cardia and gastric adenocarcinoma. The studied population was randomized to either surgery alone (23 patients) or to surgery with adjuvant treatment [intravenous (*iv*) 5-fluorouracil plus radiotherapy for 4-5 wk: 39 patients]. The 5 year survival rate was 23% in the experimental group, while it only reached

4% in controls ($P < 0.052$). Patients of the experimental group had a significantly longer postoperative disease-free period ($P = 0.02$) and lived longer than controls ($P = 0.012$). A lower incidence of loco-regional recurrence in experimental group was observed (39% *vs* 54%), but it did not reach statistical significance. The British Stomach Cancer Group published preliminary and final data of a RCT, in 1989[45] and in 1994[46], respectively, which included 436 patients resected for gastric carcinoma, randomized to either receive surgery alone (145 patients), surgery with radiotherapy (4500-5000 Gy in 25 fractions, 153 patients), or surgery followed by chemotherapy (*iv* 5-fluorouracil, adriamicin, mitomycin C given intravenously, for eight cycles: 138 patients). The tumors were at stage Ⅱ-Ⅳ, in stage Ⅳ were included also local non-radical resections. The median time to randomization was 13 d, with a range of 1-81 d. The chemotherapy protocol was completed by 42% of patients of that group, while the radiotherapy protocol was completed by 102 of the 117 patients (87.2%) who had been randomized for adjuvant radiotherapy. The protocols were not completed for the following reasons: worsening postoperative health status, withdrawal of consent, gastrointestinal, hematological and biochemical alterations due to the adjuvant treatments. The median overall survival was 15 mo, significantly influenced by the stage of primary lesion ($P < 0.0001$). The overall survival did not differ significantly in the three randomized groups ($P = 0.07$), and the 5-year-survival of the two experimental groups was not significantly different from that of the control group. Clinical recurrence in the area of the stomach or in regional lymph nodes was significantly lower ($P < 0.01$) in the two experimental groups. The milestone of adjuvant chemoradiotherapy treatment, however, is the multicentric RCT of Intergroup 0116 (SWOG 9008)[42], whose data were published in 2001. This study examined 556 patients who received curative surgery [only R0 resections were considered, while macroscopic (R2) and microscopic (R1) residual disease in relation to surgical treatment or to resulting pathology report of the removed specimen were excluded] for gastric carcinoma at stage IB-IVM0[48]. AD2 lymphadenectomy was recommended, but it was performed in 10% of the cases, 36 % had a D1 dissection, and 54% had a D0 lymphadenectomy (not all perigastric lymph nodes were removed). After gastrectomy 281 patients were randomized for experimental adjuvant treatment with chemotherapy (*iv* 5-fluorouracil and leucovorin) and loco-regional radiotherapy (area of the stomach bed and regional lymph nodes, 4500 cGy), while the other 275 patients received surgery alone. Of the 281 patients assigned to the experimental group, 64% completed the protocol treatment, while 17% did not, because of hematological and gastrointestinal side effects. Other reasons for suspending treatment were disease progression and withdrawal of consent. Before and after radiotherapy deviations from protocol were observed in 40% of cases. After a median follow-up period of 5 years, the median survival period in the experimental group was 36 mo, compared to 27 mo in controls. The 3-year survival rate was higher in the experimental group (50% *vs* 41%). The HR for death in controls, compared to the experimental group, was 1.35 ($P = 0.005$); the HR for recurrence was also higher in the control group than in the experimental group: 1.52 ($P < 0.001$). The median duration of recurrence-free survival was 30 mo in the experimental group and 19 mo in controls. The 3-year recurrence-free survival rate was 48% in the experimental group and 31% in controls. Recurrence was observed in 64% of patients in the control group and in 43% of patients in the experimental group. The incidence of both local and regional recurrence was lower in the experimental group (19% *vs* 29% and 65% *vs* 72%, respectively). The study showed that postoperative loco-regional radiotherapy and systemic chemotherapy significantly improved both overall and recurrence-free survival. In relation to these results, adjuvant chemoradiotherapy for gastric carcinoma has become common, although the Intergroup 0116 trial was criticized as the adjuvant treatment was considered a form of "compensation" for the type of employed lymphadenectomy. This criticism seems justified, considering the results of a retrospective study published in 2010[49] which examined survival and recurrence data of 91 patients receiving macroscopic radical gastrectomy with at least D1 lymphadenectomy (perigastric lymph nodes), for gastric carcinoma at stage I b-Ⅳ (AJCC)[50] followed by radiotherapy (on gastric area, anastomosis, and regional lymph nodes) combined with different chemotherapy schedules (fluorouracil and leucovorin, capecitabine alone or capecitabine and cisplatin). The control group included 694 patients from the Dutch Gastric Cancer Group Trial[25,36] who were randomized between D1 (369 patients) and D2 lymphadenectomy (325 patients). The characteristics of the studied population differed significantly in sex, age, parietal extension of the tumor, lymph node involvement, histological type of tumor, radicality of surgery, and extension of lymphadenectomy. At the time of analysis, the median follow-up for the experimental group was 19 mo, while that of controls was 51 mo. Over a 24-mo period, local recurrence was significantly lower in the experimental group (HR = 3.23; $P = 0.0015$). This was especially due to the high incidence of local recurrence after D1 resections in controls, compared to D1 resections of the experimental group (HR = 11.1; $P = 0.001$). The D2 resections in the experimental group and in the controls showed no significant differences in terms of local recurrence. Survival at 24-mo was not significantly different in the experimental group and in the controls, both overall and in D1 and D2 resections. The outcomes were significantly better in the experimental group than in controls in relation to overall survival at 24 mo after R1 resection (HR = 2.91; $P = 0.002$) and to local recurrence after R1 (HR = 5.36; $P = 0.02$) and after R0 (HR = 2.53; $P = 0.03$).

The results of an observational study on the efficacy of adjuvant chemoradiotherapy after D2 gastrectomy for operable gastric carcinoma were published in 2005[51].

The experimental group included 544 patients radically resected (R0), receiving the same adjuvant treatment performed in Intergroup 0116 trial (SWOG-9008)[42]. The controls comprised 446 patients who received the same surgery as the experimental group. In the experimental group there was a higher incidence of undifferentiated carcinomas ($P = 0.0021$), and of stage ⅢA ($P = 0.005$) and stage Ⅳ ($P = 0.0011$) (AJCC)[50] carcinomas than in controls. The treatment protocol was completed in 75.2% of the experimental cases. Forty three percent of patients in the experimental group and 49.8% in the control group had died at a median follow-up of 66 mo. The median duration of overall survival in the experimental group was significantly longer than in controls (95.3 mo *vs* 62.6 mo), with a HR for death of 0.80 ($P = 0.02$) in the experimental group, which entails a 20% reduction of the death risk. Five-year survival was significantly higher in the experimental group than in controls (57.1% *vs* 51%; $P = 0.01$). The survival benefit of adjuvant treatment was observed for all stages of gastric carcinoma and the average duration of recurrence-free survival was higher in the experimental group (75.6 mo *vs* 52.7 mo; HR for recurrence 0.80; $P = 0.016$). The probability of recurrence-free survival at 5 years was 54.5% in the experimental group and 47.9% in controls ($P = 0.01$). The HR for recurrence was better in the experimental group for all stages of gastric carcinoma, and in both groups distant recurrences prevailed (37.7%). The incidence of loco-regional recurrence within the radiation field was lower in the experimental group than in the surgery-alone group (14.9% *vs* 21.7%; $P = 0.005$). The Intergroup 0116 (SWOG 9008) in 2009[52] and in 2012[53] published the updated results of the earlier 2001 RCT[42] at a median follow-up of more than 10 years. The HR for overall survival and the HR for relapse-free survival were still better in the experimental group (HR = 1.32, $P = 0.004$; HR = 1.51, $P = 0.001$ respectively) than in controls. Recurrence rates were significantly lower ($P < 0.001$) in the experimental group. Furthermore, diffuse histotype tumors, which are more frequent in women, had lower response to adjuvant treatment. A meta-analysis published in 2007[54] was aimed to determine if there was any benefit of employing adjuvant chemoradioterapy, compared to surgery alone. The five RCTs analyzed included 868 patients, 444 in the experimental group and 424 in the controls. The adjuvant treatment protocol was not completed in 26.7% of patients due to hematological and gastrointestinal toxicity. The experimental group showed a 5-year OR for mortality significantly lower than the control group (0.45; $P = 0.00001$). The authors comment that the benefits of adjuvant chemoradiotherapy treatment may outweigh the risks in patients with a high probability of local and distant recurrence, while the risks outweigh the benefits in patients with low probability of local and distant failure. The results of a multicentric trial of the Adjuvant Chemoradiation Therapy in Stomach Cancer (ARTIST)[55] were published in 2011. The study included 458 patients who received curative D2 gastrectomy for cancer and randomized to receive two types of adjuvant treatment after surgery. One group (226 patients) was assigned to receive chemotherapy (capecitabine and cisplatin for 6 cycles), while the other group (230 patients), was assigned to receive chemoradiotherapy (capecitabine and cisplatin for 2 cycles, then radiotherapy with capecitabine for 5 wk and capecitabine and cisplatin for 2 cycles). The adjuvant protocol was completed by 75.4% of patients in the chemotherapy group and by 81.7% of patients in the chemoradiotherapy group. Gastrointestinal and hematological toxicity of grade 3-4 was observed in both groups with similar rates, while neutropenia was more frequent in the chemioradiotherapy group (43.6% *vs* 35%). After a median follow-up of 53.2 mo, these authors observed 72 cases of recurrence in the chemotherapy group and 55 cases in the chemoradiotherapy group (P = NS). No significant statistical differences were observed in loco-regional and distant recurrence rates within the two populations. The 3-year disease-free survival rates were 78.2% in the chemoradiotherapy group and 74.2 in the chemotherapy group ($P = 0.086$).

In relation to patients with lymph node metastases, disease-free survival was longer in the chemoradiotherapy than in the chemotherapy group (77.5% *vs* 72.3%, $P = 0.035$). At multivariate analysis, the duration of stage-adjusted disease-free survival was positively influenced by chemoradiotherapy in cases with lymph node metastasis (HR = 0.68, 95%CI: 0.47-0.99; $P = 0.04$). Main data regarding adjuvant chemoradiotherapy for gastric cancer are shown in Table 4.

Adjuvant chemotherapy: The clinical trials of adjuvant chemotherapy for gastric carcinoma have a long history, therefore many drug regimens have been studied, but few report evidenced survival benefit[56-58]. One of the main large-scale RCTs (more than 500 patients), in which adjuvant chemotherapy was tested in patients receiving curative surgery for gastric cancer (stage Ⅱ, ⅢA, ⅢB[10]) was examined, was published in 2007 by ACTS-GS group[59]. In this multicentric study 529 patients were randomized to receive adjuvant chemotherapy (S-1, an oral fluoropyrimidine, 6-wk cycles for one year), while 530 patients were treated by surgery alone. Patients of both groups were followed up for 5 years after surgery. The first interim analysis, conducted one year after enrollment of the last patient, at a median follow-up of 3 years, showed that the chances of overall and relapse-free survival of the experimental group might be significantly higher than those of controls and close to the predetermined threshold value ($P < 0.001$). Therefore the trial was closed. Adverse events of grade 3-4 were observed in both groups, although with higher rates in the experimental group. Sixty five percent of patients of the experimental group completed the treatment, but for 46.5% of them the chemotherapy dosage was reduced. Reasons for the interrupting adjuvant treatment included withdrawal of consent, complications, disease progression. The HR for death and for recurrence in the experimental group

Table 4 Main data regarding adjuvant chemoradiotherapy for gastric cancer

Ref.	Study design	No. of patients	Survival (*P* value)	Disease free survival (*P* value)	Recurrence (*P* value)	Loco-regional recurrence (*P* value)
Moertel *et al*[44], 1984	RCT surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT)	S: 39 SCRT: 23	At 5 yr S < SCRT (0.05)	At 5 yr S < SCRT (0.02)	-	S *vs* SCRT (NS)
Allum *et al*[45], 1989 BSCG	RCT surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT) *vs* surgery + chemotherapy (SCT)	S: 145 SCRT: 153 SCT: 138	At 5 yr S *vs* SCRT *vs* CT (NS)	-	-	S *vs* SCRT (NS) S *vs* SCT (NS)
Hallissey *et al*[46], 1994 BSCG	RCT surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT) *vs* surgery + chemotherapy (SCT)	S: 145 SCRT: 153 SCT: 138	At 5 yr S *vs* SCRT *vs* CT (NS)	-	-	-
Macdonald *et al*[42], 2001 INT-0116	RCT surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT)	S: 275 SCRT: 281	At 3 yr S < SCRT (0.005)	At 3 yr S < SCRT (0.001)	At 3 yr S > SCRT (0.001)	At 3 yr S > SCRT (0.0001)
Dikken *et al*[49], 2010	Retrospective surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT)	S: 694 SCRT: 91	At 24 mo S *vs* SCRT (NS)	At 24 mo S *vs* SCRT (NS)	-	At 24 mo S > SCRT (0.0015)
Kim *et al*[51], 2005	Observational surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT)	S: 446 SCRT: 544	At 5 yr S < SCRT (0.01)	At 5 yr S < SCRT (0.01)	-	At 5 yr S > SCRT (0.005)
Smalley *et al*[53], 2012 INT-0116	RCT surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT)	S: 227 SCRT: 282	At 10 yr S < SCRT (0.0046)	At 10 yr S < SCRT (0.001)	At 10 yr S > SCRT (0.006)	Ar 10 yr S > SCRT (0.0001)
Fiorica *et al*[54], 2007	Meta-analysis surgery alone (S) *vs* surgery + chemoradiotherapy (SCRT)	S: 424 SCRT: 444	At 5 yr S < SCRt (0.00001)	-	-	-
Lee *et al*[55], 2011	RCT surgery + chemoradiotherapy (SCRT) *vs* surgery + chemotherapy (SC)	SCRT: 230 SC: 228	-	At 3 yr SCRT *vs* SC (NS)	SCRT *vs* SC (NS)	SCRT *vs* SC (NS)

RCT: Randomized controlled trial; NS: Not significant.

compared to those of controls were 0.68 ($P = 0.003$) and 0.62 ($P = 0.001$), respectively. The 3-year overall survival rates were 80.1% in the experimental group and 70.1% in controls ($P = 0.003$). The 3-year recurrence-free survival rates were 72.2% in the chemotherapy group and 59.6% in the surgery-alone group ($P < 0.001$). The rates of nodal and peritoneal recurrence were higher in the control group ($P = 0.006$). The 5-year update of this trial[60] confirmed the benefits of adjuvant chemotherapy compared to surgery alone in terms of 5-year survival (71.7% *vs* 61.1%), HR for death (HR = 0.66, 33.1% reduction of death risk in the experimental group), 5-year recurrence-free survival rate (65.4% *vs* 53.1%), HR for recurrence (HR = 0.65, 34.7% reduction of recurrence risk in the experimental group). The percentages of five-year overall and recurrence-free survival were analyzed according to the tumor stage (Ⅱ, ⅢA, ⅢB),and proved to be better in the experimental group than in controls (HR between 0.50 and 0.79). An Asiatic multicentric RCT, whose results were published in 2012[61], examined the effects of adjuvant chemotherapy on disease-free survival (oral capecitabine and *iv* oxaliplatin, for 8 cycles in 6 mo) compared to surgery alone. Of the 1035 patients of the study, all receiving radical surgery and D2 lymph node dissection for gastric cancer at stage Ⅱ-ⅢB[10], 515 were randomized to receive surgery alone, 520 to receive adjuvant chemotherapy. Sixty seven percent of patients in the experimental group completed treatment. Adverse events of grade 3-4 were nine times more common in the experimental group and required reduction of dosage, delay in administration, or interruption of chemotherapy. Also this trial showed the benefits of adjuvant chemotherapy compared to surgery alone in terms of 3-year disease-free (74% *vs* 59%, HR = 0.56; $P < 0.0001$), overall survival (83% *vs* 78%, HR = 0.72; $P = 0.049$), recurrence or new occurrences of gastric cancer (18% *vs* 30%), loco-regional recurrence (21% *vs* 44%). The benefits of adjuvant chemotherapy on 3-year disease-free survival were clear even when analyzed according to stage of the tumor, but not for patients without lymph node metastases. The results of two meta-analyses assessing if patients with gastric cancer could benefit from chemotherapy after curative resection were published in 2009[62] and in 2010[63]. These researches considered 12 RCTs, with a total of 3809 patients[62], and 17 RCTs, with a total of 3838 patients[63], respectively. Over 60% of the patients in the experimental group completed the treatment protocol[62]. The overall survival rates at 5 and 10 years were higher in the adjuvant chemotherapy groups than in controls, even if the differences were not significant in all the RCTs. In the two meta-analyses the HR for death was significantly better (between 0.78 and 0.82) ($P < 0.001$), with a reduction of the death risk ranging from 18% to 22% in the experimental group. Estimated median over-

all survival was 4.9 years in the surgery-alone group and 7.8 years in the adjuvant chemotherapy group. The rates of absolute benefits in the experimental group were 5.8% (55.3% *vs* 49.6%) at 5 years, and 7.4% (44.9% *vs* 37.5%) at 10 years,compared to the surgery-alone group[63]. Adjuvant chemotherapy better influenced disease-free survival than surgery alone, with a HR of 0.82 ($P < 0.001$). The rate of absolute benefit for five-year disease-free survival after adjuvant chemotherapy was 5.3 (54.0% *vs* 48.7%) compared to surgery alone[62]. With regard to the various chemotherapy regimens (mono chemotherapy and polychemotherapy) used in the RCTs examined in the two meta-analyses, 5-fluorouracil was used in all the studies, and anthracycline and mitomycin C were used along with it in various trials. Adjuvant monochemotherapy showed a significant benefit in 5-year survival compared to surgery alone (71.4% *vs* 53.9%, HR = 0.6; $P = 0.03$), with better results compared to adjuvant polychemotherapy[63]. The data of these two meta-analyses confirm those of another meta-analysis dated 2008 in terms of survival rate and disease-free survival[64]. In addition, the latter study proved a positive influence of adjuvant therapy in relation to loco-regional and distant recurrence rate (RR = 0.78).

The main disadvantage of adjuvant treatments, both chemotherapy and chemoradiotherapy, is that these cannot be performed before post-surgery healing is complete and the general conditions of the patients are satisfactory. Therefore, postoperative complications or slow recovery after surgery can delay the beginning of the treatment. Due to the anatomic conditions created by surgery, radiotherapy can affect other organs and possibly cause irradiation damage. Chemotherapy can often cause negative effects or toxicity in the gastrointestinal system of patients whose new anatomical gastrointestinal conditions created by surgery often cause functional alterations. Main data regarding adjuvant chemotherapy for gastric cancer are shown in Table 5.

Neoadjuvant treatments

The aim of neoadjuvant treatments is to reduce the biological potential of tumor cells, to increase surgical radicality, and to eradicate subclinical micrometastases. The advantages of neoadjuvant treatments lie in the fact that patients who receive these are in good health condition, the treatment can start immediately after diagnosis and clinical staging are conducted. If radiotherapy is performed, the treatment is aimed at the target organ that, anyway, will be resected, while if chemotherapy is performed, the possible gastrointestinal side effects are not worsened by the anatomical and functional alterations that may occur after surgery. Also, the downsizing of the neoplasm after neoadjuvant treatment can facilitate surgery. For these reasons, the neoadjuvant treatment seems attractive and can be administered to more patients than adjuvant treatment. The disadvantage of neoadjuvant treatment is that it delays surgery, with the possibility that the tumor will extend beyond the stage of resectability or that the health conditions after the neoadjuvant treatment will not allow for surgery[65]. For these reasons, the studies which have been examined report a limited number of patients because of difficulty of enrollment or early closure of the trials, with consequences on the quality of the studies.

Neoadjuvant chemotherapy: The Dutch Gastric Cancer Group in 2004 published the long-term results of the RCT FAMTX[66], which examined the effect of preoperative chemotherapy (methotrexate, 5-fluorouracil, leucovorin and doxorubicin every four weeks for 4 cycles) in patients with gastric carcinoma, in terms of resectability and survival. After randomization, the analysis was conducted on 27 patients in the experimental group (neoadjuvant chemotherapy followed by surgery) and on 29 controls (surgery alone). The interval between randomization and surgery in the experimental group was significantly longer than in controls ($P < 0.001$). Forty four percent of patients in the experimental group interrupted chemotherapy because of toxicity. Lymphadenectomy was limited to perigastric lymph nodes (D1) in both groups. The rate of curative resections (R0) was similar in both groups. The median postoperative follow-up for the two groups was 83 mo. The median survival after randomization was 18.2 mo in the experimental group and 30.3 mo in controls. The 5-year survival rate was 21% in the experimental group and 34% in controls ($P = 0.17$). The last data confirmed a trend to adverse effects due to preoperative chemotherapy, although not significant. The survival rates of patients receiving curative surgery (R0) were 32% in the experimental group and 53% in controls. The trial was closed after the enrollment of 59 patients and after an interim analysis showed inadequate rates of curative resections in the experimental group. The results of a RCT of the European Organization for Research and Treatment of Cancer were published in 2010[67]. This study compared neoadjuvant chemotherapy and surgery alone in patients with gastric and cardia adenocarcinoma, at clinical stage UICC Ⅲ and Ⅳ, cM0. Patients were randomized in experimental group (72 patients) treated with neoadjuvant chemotherapy (*iv* cisplatin, folinic acid, fluorouracil, 2 cycles of 48 d), and controls (72 patients), only receiving surgery. Sixty two percent of patients in the experimental group completed neoadjuvant treatment. Reasons for interruption of treatment were: toxicity, withdrawal of consent and progression of the disease. Surgical resection was performed within 14 d from randomization in controls (68 patients), and within four weeks from last day of chemotherapy in the experimental group (70 patients). D2 gastrectomy was performed in the majority of patients. In the experimental group, the tumor had smaller dimensions than in controls, and in both groups a complete resection was possible in 87.5% of cases. Postoperative morbidity was more frequent in the experimental group ($P = 0.09$), operative mortality was observed in one patient of the controls and in two patients of the experimental group.

Table 5 Main data regarding adjuvant chemotherapy for gastric cancer

Ref.	Study design	*n*	Survival (*P* value, HR, RR)	Disease free Survival (*P* value, HR, RR)	Recurrence (*P* value)	Loco-regional Recurrence (*P* value)
Sakuramoto *et al*[59], 2007	RCT Surgery alone (S) *vs* surgery + chemoterapy (SC)	S: 530 SC: 529	At 3 yr S < SC (0.003)	At 3 yr S < SC (0.001)	At 3 yr S > SC (0.001)	At 3 yr S > SC (0.006)
Sasako *et al*[60], 2011	RCT Surgery alone (S) *vs* surgery + chemoterapy (SC)	S: 530 SC: 529	At 5 yr SC benefit *vs* S HR = 0.66	At 5 yr SC benefit *vs* S HR = 0.65	At 5 yr S > SC (0.008)	At 5 yr S > SC (0.005)
Bang *et al*[61], 2012	RCT Surgery alone (S) *vs* surgery + chemoterapy (SC)	S: 515 SC: 520	At 3 yr SC benefit *vs* S HR = 0.72 (0.049)	At 3 yr SC benefit *vs* S HR = 0.56 (0.0001)	At 3 yr S > SC (0.0006)	At 3 yr S > SC (0.0003)
Liu *et al*[64], 2008	Meta-analysis Surgery alone (S) *vs* surgery + chemoterapy (SC)	S: 2313 SC: 2286	At median 5 yr SC benefit *vs* S RR = 0.85 (0.00001)	At median 5 yr SC benefit *vs* S RR = 0.85 (0.04)	At median 5 yr SC benefit *vs* S RR = 0.78	At median 5 yr SC benefit *vs* S RR between 0.62-0.65
Sun *et al*[62], 2009	Meta-analysis Surgery alone (S) *vs* surgery + chemoterapy (SC)	S: 1914 SC: 1931	At 5 yr SC benefit *vs* S HR = 0.78 (0.001)			
Paoletti *et al*[63], 2010	Meta-analysis Surgery alone (S) *vs* surgery + chemoterapy (SC)	S: 1885 SC: 1953	At 10 yr SC benefit *vs* S HR = 0.82 (0.001)	At 10 yr SC benefit *vs* S HR = 0.82 (0.001)		

RCT: Randomized controlled trial.

In the experimental group, a complete clinical response was obtained in 5.8% of patients, while a partial clinical response was obtained in 30.4%. Following pathological assessment of the operative specimen, 81.9% of patients in the experimental group had received a radical resection (R0), compared to 66.7% of controls ($P = 0.036$). In the experimental group, complete pathological response was observed in 7.1% of the patients, and 65.7% of tumors in the experimental group were T0-1-2, which compares to 50% in controls. Lymph node metastases and lymphatic invasion were significantly higher in controls ($P = 0.018$ and $P = 0.01$ respectively). At a median follow-up of 4.4 years, however, no significant survival benefit was observed in the experimental group (HR for overall survival 0.84; $P = 0.46$). In a multicentric RCT, published in 2010[68], the short-term effects of neoadjuvant chemotherapy (docetaxel, cisplatin, 5-fluorouracil for 4 cycles of 21 days) in 34 patients with gastric carcinoma (T3-4 any N M0 or any T N1-3 M0 TNM 1997) were examined, comparing them with the same chemotherapy in adjuvant treatment (in 35 patients). Patients in the neoadjuvant group received surgery 3-4 wk after beginning the last cycle of therapy. Neoadjuvant therapy was completed in 74% of patients of that group. D1 lymphadenectomy was always performed, and it was sometimes extended to some stations of the main local vessels[10]. Although it is not possible to compare the two groups, because of the characteristics of the study, radical R0 resection was performed in 85% of cases in the neoadjuvant group and in 91% of the adjuvant group. Complete pathological response was observed in 12% of cases in the neoadjuvant group. Postoperative complications and operative mortality did not differ significantly ($P = 0.86$) in the two groups. Complete adjuvant therapy was administered to 34% of patients in the adjuvant group. Thirty four percent of patients in this group did not receive adjuvant treatment. Severe adverse events were more frequent in the adjuvant group ($P = 0.07$).

D'Ugo *et al*[69], based on a study with a single treatment group of 34 patients with resectable gastric carcinoma, state that the postponement of resection in favour of a systemic treatment does not exclude patients from the benefits of a potentially curative delayed resection and does not worsen surgical outcomes. However, they also admit that tumor progression affects some patients. This statement alone would be enough to suggest an accurate selection of those patients who can benefit from preoperative chemotherapy.

Pre- and post-operative chemotherapy (perioperative chemotherapy): This approach presents the disadvantages of both modalities. Besides, a percentage of patients does not start postoperative treatment due to progression of the disease, toxicity during preoperative treatment, withdrawal of consent, or postoperative complications.

The results of an international multicentric RCT were published in 2006[43] by Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC), and it has become a landmark for all the following studies on neoadjuvant and adjuvant treatments for gastric carcinoma. The study examined 503 patients with gastric, esophagogastric junction and distal esophageal adenocarcinoma, amenable to curative surgery. Seventy four percent of the examined cases were gastric carcinomas. Patients were randomized to receive perioperative chemotherapy (250 patients, three preoperative cycles and three postoperative cycles of epirubicin, cisplatin and fluorouracil for 21 d) or surgery alone (253 patients). Eighty six percent of patients in the experimental group (215 patients) com-

pleted neoadjuvant treatment, and toxic effects of the therapy were the main reasons for interruption. Two hundred and twenty nine patients of the experimental group were resected. One hundred and four of them completed the three cycles of pre- and post-operative chemotherapy. The main reasons for not performing postoperative therapy were: progression of the disease, death, withdrawal of consent. The median interval between randomization and surgery was 99 d in the experimental group and 14 d in controls. Smaller maximum diameter of the tumor, higher number of T1-T2 tumor and smaller lymph node involvement were observed in the experimental group, with significant differences as compared to controls (P = 0.001, P = 0.002, P = 0.01, respectively). The percentages of death, death for progression of the disease, local and distant recurrence were higher in controls. The HR for progression risk and for death in the experimental group was better than in the control group (HR = 0.66; P = 0.001 and HR = 0.75; P = 0.009 respectively). Even after stratification, HR for death was better in the experimental group (0.74; P = 0.008). The percentages of survival at 5 years were 36.3% in the experimental group and 23% in controls.

The results of the French multicentric RCT FLNCC ACCORD07-FFCD 9703 were published in 2011[70]. This research compared pre- and postoperative chemotherapy in patients with resectable gastric, esophagogastric junction and distal esophagus adenocarcinoma (cisplatin, fluorouracil; 2-3 preoperative cycles and 3-4 postoperative cycles of 28 d) with surgery alone. One hundred and thirteen patients were randomized in the experimental group, 111 in the controls. Seventy five percent of the adenocarcinomas were in the distal esophagus or in the esophagogastric junction. Eighty seven percent of patients in the experimental group received at least 2 cycles of preoperative chemotherapy. Toxicity of grade 3-4 developed during preoperative therapy in 38% of patients in the experimental group. Surgery was performed in 96.5% of cases in the experimental group, at a median interval of 78 d after randomization, and in 99% of controls, at a median interval of 13 d after randomization. R0 resections and a lower incidence of lymph node metastases were found in the experimental group (P = 0.04 and P = 0.054 respectively). Fifty percent of patients in the experimental group received postoperative chemotherapy. At a median follow-up of 5.7 years, the experimental group showed a significant benefit in terms of overall (HR for death 0.69; P = 0.02) and disease-free survival (HR = 0.65; P = 0.003) compared to controls. The 5-year survival rates were 38% in the experimental group *vs*, 24% in controls. The disease-free survival rates were 34% and 19%, respectively. At multivariate analysis, preoperative chemotherapy was a significant prognostic factor (P = 0.01). The results of this trial, in relation to gastric carcinoma, need to be cautiously assessed, considering that gastric adenocarcinomas were only 25% of all the adenocarcinomas.The study protocol of an international multicentric RCT, CRITICS (ChemoRadiotherapy after induction Chemotherapy in Cancer of the Stomach, NCT00407186), was published in 2011[71]. According to this study, patients with a resectable gastric cancer (stage IB-IVa AJCC 6th edition) should be treated with three cycles of preoperative chemotherapy (epirubicin, cisplatin, capecitabine) followed by surgery with D2 lymphadenectomy, then, following these, three more cycles of the same chemotherapy or chemoradiotherapy. The number of patients to be enrolled is high, 788 patients. These should be randomized after diagnosis and before beginning neoadjuvant treatment. The primary endpoint is to improve overall survival compared to surgery alone. It is expected that the results of this trial will be useful to treat patients with resectable gastric cancer.

NEOADJUVANT AND INTRAOPERATIVE RADIOTHERAPY

The aim of neoadjuvant and intraoperative radiotherapy is to reduce the biological potential of tumor cells, to increase surgical radicality by reducing the extension of the tumor, and to eliminate residual subclinical local metastases after surgery. The surgeons, however, are particularly concerned about the technical difficulties of operating in a pretreated field, because of the possibility of wound anastomotic healing problems, damage to nearby abdominal organs, and postoperative pulmonary complications. Also for these reasons, these methods of treatment are not common, and there are few trials examining their efficacy. In addition, these techniques also bear logistic difficulties, especially in relation to intraoperative radiotherapy.

The short- and long-term results of a RCT were published in 1998[72] examining the role of neoadjuvant radiotherapy compared to surgery alone, in the treatment of gastric cardia adenocarcinoma. After randomization, the experimental group included 171 patients, compared to 199 controls. The radiation dose was 40 Gy, and it was administered in 4 wk. Surgery was performed 2-4 wk after completion of radiotherapy. The rates of overall, radical and palliative resectability were significantly higher in the experimental group (P = 0.01, 0.001, 0.025 respectively). The experimental group presented a smaller local extension of the tumor, a lower number of patients with lymph node metastases and a lower number of lymph nodes affected by metastases (P < 0.01, P < 0.001 and P < 0.0001 respectively). Preoperative radiotherapy obtained better overall 5- and 10-year survival rates, both in resected and in non-resectable patients, while the difference compared to surgery alone was only significant (P = 0.009) in the latter. Non-resectable patients in the experimental group had significantly longer mean and median survivals (P = 0.008) than non-resectable patients in controls. After histological examination, it was observed that a higher effect of radiotherapy on tumor cells corresponded to better 5-year survival rates (P = 0.05). Local and lymph node recurrences were lower in the experimental group (P < 0.025 and P < 0.005 respectively)

than in controls. It should be considered that the examined cases were adenocarcinoma of gastric cardia, which can account for the positive results. The data of a RCT of Russian MRRC RAMS were published in 2000[73]. It randomized 40 patients with gastric carcinoma to receive concentrated preoperative radiotherapy (20 Gy in 5 d), gastrectomy and intraoperative radiotherapy (IORT, 20 Gy) and 38 patients to receive gastrectomy alone. The use of IORT allows for a higher administration of radiations directly on a target area and, together with concentrated adjuvant radiotherapy, it has the theoretical potential to reduce local and regional recurrence and to improve survival. D1 gastrectomy was prevalently performed in the two groups. In 25% of cases neoadjuvant radiotherapy caused toxicity in the experimental group, but the preoperative radiotherapy treatment was always completed. Postoperative complications were observed in 35% of cases in the experimental group and in 50% in the control group. Overall survival was not significantly different in the two groups ($P = 0.31$). However, in the experimental group a benefit in survival was observed, when lymph nodes were metastasized ($P = 0.04$), when tumors were T3-4 ($P = 0.04$), and for stage Ⅱ and Ⅲ A tumors ($P = 0.04$). This trend was also seen in T3-4 N1-2 cases: median survival time was 21.4 mo in the experimental group, 9.0 mo in controls. These data confirm that, for T1-2 N0 stage tumors, neoadjuvant and adjuvant therapies do not improve the results of surgery, which, in these particular instances, is ideal and sufficient. The data of another RCT of the MRRC RAMS were published in 2002[74]. This research examined the short- and long-term outcomes of neoadjuvant radiotherapy employed in resectable gastric carcinomas. Fifty one patients were randomized in the experimental group and treated with concentrated preoperative radiotherapy (20 Gy administered in 5 consecutive days) followed by surgery within 4-5 d. The 51 controls received surgery within 7 d from randomization. Radiotherapy, completed by all patients in the experimental group, was generally well tolerated. D1 gastrectomy was always performed, and postoperative (surgical and non-surgical) complications were more frequent in the experimental group,while operative mortality did not differ in the two groups. Long-term survival in the two groups was not significantly different (39% in the experimental group *vs* 30% in controls after 5 years; 32% in the experimental group *vs* 18% in the control group after 10 years) ($P = 0.55$), although median duration of survival of tumors T3-4 and of tumors with positive lymph nodes was longer in the experimental group. The survival curves in the experimental group became better than in controls three years after surgery. The authors comment that the long-term aim of radiotherapy is to reduce the development of loco-regional recurrence, and the trend of the curves confirmed that this result had been achieved. A meta-analysis was conducted in 2007[54], examining four RCTs for a total of 405 patients treated with adjuvant radiotherapy for resectable gastric carcinoma, and it showed a benefit of radiotherapy after 3 and 5 years compared to surgery alone (OR = 0.57; $P = 0.0001$ and OR = 0.62; $P = 0.002$, respectively). One other meta-analysis, in 2009[75], also considered the role of neoadjuvant and adjuvant intraoperative radiotherapy in gastric carcinoma. The modalities of the analysis, however, do not allow to understand which results refer to the various types of radiotherapy.

Intraperitoneal chemotherapy

Negative outcomes after surgery for gastric carcinoma are related, in 50% of the cases, to dissemination of the tumor in the peritoneal cavity[9,76]. The peritoneum is involved either as a result of trans-parietal invasion by tumor cells, or of intraperitoneal seeding caused by surgical maneuvers such as manipulation of the tumor or section of lymphatic and blood vessels. Chemotherapy medications injected during neoadjuvant or adjuvant therapies do not reach effective concentration at peritoneal level. Intraperitoneal chemotherapy aims at eradicating tumor cells after surgery by using high concentrations of drugs in the peritoneum, thus reducing systemic toxicity. The use of intraperitoneal chemotherapy can be considered a procedure with prophylactic and therapeutic aim, but, to date, it is not accepted as a standard therapy[77]. It can be given in several ways: as hypertermic intraoperative intraperitoneal chemotherapy (HICC), as normothermic intraoperative intraperitoneal chemotherapy (NIIC), as early postoperative chemotherapy (EPIC) or as delayed postoperative intraperitoneal chemotherapy (DPIC). Some problems are still unsolved: the short- and long-term outcomes of intraperitoneal chemotherapy, the correct timing, the role of hyperthermia, the drugs to be employed, and, more importantly, the selection of patients to be treated. Since the '90s, several RCT have been conducted, especially by Eastern authors, in order to examine the role of intraperitoneal chemotherapy in gastric carcinoma, with the use of cisplatin, mitomycin C, 5-fluorouracil alone or in combination, comparing intraperitoneal chemotherapy to surgery alone[78-80]. The results of these studies are ambiguous. A RCT in 2001[80] showed a survival benefit in patients with resection of stage Ⅳ and stage Ⅲ gastric cancer followed by intraperitoneal chemotherapy (mitomycin C and 5-fluorouracil), compared to surgery-alone controls, while there was no significant benefit in patients with stage Ⅰ or Ⅱ. The 5-year survival for patients with stage Ⅲ and stage Ⅳ disease were 57% and 28% in the treated group, and 23% and 5% in the surgery-alone controls, respectively ($P = 0.0024$ and $P = 0.0098$). The benefits of intraperitoneal chemotherapy on survival were significant as compared to controls in tumors with gross serosal invasion ($P = 0.0004$), lymph node metastases ($P = 0.0027$), in tumors > 5 cm in diameter ($P = 0.0029$), and in poorly differentiated tumors ($P = 0.0004$). Furthermore, the incidence of peritoneal dissemination in the treated group was 15%, compared to 30% after surgery alone ($P = 0.03$). A significant benefit in terms of long-term survival ($P = 0.03$) and peritoneal recurrence ($P = 0.00008$) of pa-

tients who received gastrectomy for gastric carcinoma with macroscopic serosal invasion had been observed in a previous RCT[81]. A meta-analysis published in 2003[82] examined the use of intraperitoneal chemotherapy in patients with gastric cancers resected with curative aim. Eight RCTs were examined, 495 patients were randomized in the experimental group, while 500 patients only received surgery. The analysis showed that intraperitoneal chemotherapy, administered during or immediately after surgery, obtained some benefit compared to surgery alone. A more recent meta-analysis, published in 2007[83] examined 13 RCTs in which patients with advanced gastric or gastroesophageal junction adenocarcinoma who received curative resection were randomized to receive surgery with associated intraperitoneal chemotherapy (873 patients), or surgery alone (775 patients). HR of the overall 3-year survival was better in the intraperitoneal chemotherapy group in all the modalities of administration, but the benefit was particularly significant in cases treated with HICC alone or with associated EPIC (HR = 0.60; $P = 0.002$ and HR = 0.45; $P = 0.0002$ respectively). The relative risk (RR) of locoregional recurrence, examined for HICC, NIIC and EPIC, did not show significant differences between experimental group and controls. The incidence of perioperative mortality of the procedures of intraperitoneal chemotherapy was not significantly different than in controls (RR = 1.03; $P = 0.96$). The most common postoperative complications in the experimental group were intra-abdominal abscess (RR = 2.37; $P = 0.003$) and neutropenia (RR = 4.33; $P = 0.007$).

CONCLUSION

Gastric carcinoma still represents one of the main causes of death for cancer in the world, although it presently has a lower incidence in Western countries. The adoption of programs of primary and secondary prophylaxis and the use of effective treatments are the only ways to tackle this condition. The optimal treatment for advanced gastric carcinoma is still controversial. Surgical gastric resection (partial or total) associated with lymphadenectomy of perigastric and regional lymph node stations (D2) represents the treatment of choice. However, surgery alone cannot guarantee satisfactory results for patients with advanced disease. The use of multimodal therapies, chemotherapy and radiotherapy, alone or combined, used as neoadjuvant, intraoperative or adjuvant treatments is supported by a series of trials substantiating their effectiveness on recurrence and survival. Timing, type of therapy, dosing of administration and possible combination between different modalities still have to be assessed and tailored in order to achieve the best outcome in the individual patient.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Treatment options in patients with metastatic gastric cancer: Current status and future perspectives

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Abstract

Despite advances in the treatment of gastric cancer, it remains the world's second highest cause of cancer death. As gastric cancer is often diagnosed at an advanced stage, systemic chemotherapy is the mainstay of treatment for these patients. However, no standard palliative chemotherapy regimen has been accepted for patients with metastatic gastric cancer. Palliative chemotherapy including fluoropyrimidine, platin compounds, docetaxel and epirubicin prolongs survival, and improves a high quality of life to a greater extent than best supportive care. The number of clinical investigations associated with targeted agents has recently increased. Agents targeting the epidermal growth factor receptor 1 and human epidermal growth factor receptor 2 (HER2) have been widely tested. Trastuzumab was the first target drug developed, and pivotal phase Ⅲ trials showed improved survival when trastuzumab was integrated into cisplatin/fluoropyrimidine-based chemotherapy in patients with metastatic gastric cancer. Trastuzumab in combination with chemotherapy was thus approved to be a new standard of care for patients with HER2-positive advanced esophagogastric adenocarcinoma. Thus, the evaluation of HER2 status in all patients with metastatic gastroesophageal adenocarcinoma should be considered. Other agents targeting vascular endothelial growth factor, mammalian target of rapamycin, and other biological pathways have also been investigated in clinical trials, but showed little impact on the survival of patients. In this review, systemic chemotherapy and targeted therapies for metastatic gastric cancer in the first- and second-line setting are summarized in the light of recent advances.

Key words: Gastric cancer; Chemotherapy; Targeted therapy; Metastasis; Advanced-stage

Core tip: Although palliative chemotherapy have been demonstrated to improve survival and quality of life, the prognosis of patients with metastatic gastric cancer remains poor and responses to first-line chemotherapy are partial and heterogeneous. In order to improve the results of currently available treatments, remarkable advancements in new targeted agents have recently been obtained. The addition of trastuzumab to cisplatin/fluoropyrimidine-based chemotherapy significantly improved survival in patients with human epidermal growth factor receptor 2-positive metastatic gastric cancer, which is now the new standard of care. Our manuscript will elucidate current systemic chemotherapy and promising targeted therapies for metastatic gastric cancer in the first- and second-line setting in the light of recent advances.

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INTRODUCTION

Gastric cancer is the second most common cause of cancer death worldwide. Although the overall incidence and mortality of this disease have dramatically declined over the last few decades, it remains a major health problem[1,2]. Radical gastrectomy is the only curative treatment of gastric cancer, but recurrences are common, being detected in approximately 60% of patients[3]. In addition, gastric cancer is often diagnosed at an advanced stage, other than in Japan and Korea, where screening is widely performed. For these patients, systemic chemotherapy is the mainstay of treatment[4,5]. Although recent phase Ⅲ studies showed some benefit from chemotherapy regimens including docetaxel, capecitabine, irinotecan, cisplatin and oxaliplatin[5], there is no internationally accepted standard of care.

Treatment responses and prognosis are highly variable even within the same stage. Therefore, a thorough understanding of cancer biology is essential for better management of gastric cancer in the future. To date, molecular targets such as epidermal growth factor (EGFR) receptor, vascular endothelial growth factor (VEGF) receptor and human epidermal growth factor receptor 2 (HER2) have been tested by clinical trials in metastatic gastric cancer[6,7]. A recent phase Ⅲ trial proved the benefit of trastuzumab (anti-HER2 antibody) in combination with chemotherapy in advanced HER2-positive gastric cancer or esophagogastric junction[8]. Despite these marked advances, the prognosis of patients with advanced gastric cancer remains poor. Therefore, new therapeutic molecular targets are required to improve the survival of patients[7].

In this article, we review the currently available treatments in light of the most recent publications and guidelines, along with promising therapeutic options that are still under development for patients with advanced gastric cancer.

CURRENT TREATMENT OPTIONS FOR ADVANCED GASTRIC CANCER

First-line chemotherapy

Palliative chemotherapy versus best supportive care (BSC) for patients with metastatic gastric cancer has been evaluated in several clinical trials, which showed that palliative chemotherapy improved overall survival (OS) for several mo longer on average than supportive care[9-12]. A meta-analysis performed by Wagner *et al*[12] demonstrated an overall HR of 0.39 (95%CI: 0.28-0.52) for OS in favor of chemotherapy compared with BSC, which translates to a benefit in weighted mean average survival of about 6 mo. Moreover, chemotherapy also provided relief of symptoms, and improved and prolonged a high quality of life more than BSC[9].

In the last 20 years, multiple randomized trials testing different combination regimens in patients with metastatic gastric cancer have indicated that there is no international consensus regarding the best management approach[13-16], and meta-analysis of these studies[12] has demonstrated that combination chemotherapy is superior to monotherapy, with a HR of 0.83 for OS (95%CI: 0.74-0.93) in favor of combination chemotherapy.

In the early 1980s, the FAM chemotherapy regimen (fluorouracil, doxorubicin mitomycin) was accepted as the gold standard regimen for patients with metastatic gastric cancer[17]. Subsequently, in a study carried out by Webb *et al*[18], 274 patients with metastatic esophagogastric cancer were randomly assigned to receive either epirubicin, cisplatin and fluorouracil (ECF) or fluorouracil, doxorubicin, and methotrexate (FAMTX). The patients treated with ECF had a significantly longer median OS (8.9 mo *vs* 5.7 mo, $P = 0.0009$) than the FAMTX group. Multiple randomized studies have compared various fluorouracil-based regimens and of all the combination regimens, ECF has been considered to be the reference standard regimen in the United States and Europe based on OS and quality of life benefits[19].

The REAL-2 trial reported that oxaliplatin and capecitabine were found to be noninferior to cisplatin and fluorouracil, with manageable toxicity profiles[20]. This trial compared capecitabine with fluorouracil and oxaliplatin with cisplatin in 1002 patients with advanced esophageal, gastroesophageal junction, or gastric cancer. In a two-by-two design, patients with histologically confirmed advanced esophagogastric cancer were randomized to receive one of four epirubicin-based regimens [ECF, epirubicin, oxaliplatin and fluorouracil (EOF), epirubicin, cisplatin and capecitabine (ECX) and epirubicin, oxaliplatin and capecitabine (EOX)]. The median OS times in the ECF, EOF, ECX and EOX groups were 9.9, 9.3, 9.9 and 11.2 mo, respectively. For the capecitabine-fluorouracil and oxaliplatin-cisplatin comparisons, the results indicated a noninferior median OS in patients treated with capecitabine rather than 5-FU (HR_{death}: 0.86; 95%CI: 0.82-0.99) and in patients treated with oxaliplatin in place of cisplatin (HR_{death}: 0.92; 95%CI: 0.80-1.10)[20]. Since REAL-2, oxaliplatin and capecitabine have often been substituted for cisplatin and 5-FU within the ECF regimen in many cancer centers.

Another phase Ⅲ randomized noninferiority trial, ML17032, performed by Kang *et al*[21], compared the combination capecitabine and cisplatin (XP) with the combination of fluorouracil and cisplatin (FP) in patients with previously untreated advanced gastric cancer in the first-line setting. Both overall response rates (ORR) and median OS times were superior for patients treated with the XP regimen (ORR; 41% *vs* 29% and OS; 10.5 mo *vs* 9.3 mo, respectively), although the median progression-free survival (PFS) time was found to be similar for both regimens (5.6 mo for XP and 5.0 mo for FP). The authors concluded that capecitabine is as effective as fluorouracil in the treatment of patients with advanced esophagogastric cancer. Thereafter, a meta-analysis of the REAL-2 and ML17032 trials demonstrated that OS was superior in the 654 patients who received capecitabine-based

regimens compared with the 664 patients treated with fluorouracil-based combinations, but there was no significant difference with respect to PFS between treatment groups[22].

An incremental improvement in OS was also suggested in the V325 trial[23]. This randomized multinational phase Ⅲ trial evaluated the combination of docetaxel, cisplatin and fluorouracil (DCF) in patients with untreated advanced gastric cancer. Four hundred and forty-five patients were randomized to receive either DCF every 3 wk or cisplatin and fluorouracil (CF). Time-to-progression (TTP) for patients who received DCF was significantly longer than that of patients treated with CF (5.6 mo *vs* 3.7 mo; HR = 1.47; 95%CI: 1.19-1.82; $P < 0.001$; risk reduction 32%). Moreover, the median OS time was significantly worse for patients who received DCF compared with patients who received CF (9.2 mo *vs* 8.6 mo; HR = 1.29; 95%CI: 1.0-1.6; $P = 0.02$; risk reduction 23%)[23]. High toxicity rates were reported in this trial, especially involving febrile neutropenia, which was more common in patients who received DCF (29% *vs* 12%); the death rate in the study was 10.4% for patients who received the DCF regimen and 9.4% for patients treated with the CF arm.

As the DCF regimen resulted in high toxicity profiles, several clinical trials have tested modifications of the DCF regimen with the aim of reducing toxicity and improving tolerability[24-26]. The recent GATE phase Ⅱ study carried out by Van Cutsem *et al*[27] showed that the combination of docetaxel, oxaliplatin and fluorouracil (DOF) had a better RR, TTP and median OS time (47%, 7.7 and 15 mo, respectively) compared with the combination docetaxel and oxaliplatin (23%, 4.5 and 9 mo, respectively) and docetaxel, oxaliplatin and capecitabine (26%, 5.6 and 11 mo, respectively) in patients with previously untreated advanced gastric cancer. Furthermore, the DOF regimen produced a better safety profile compared to other regimens.

Al-Batran *et al*[28], in their phase Ⅲ trial, reported that median PFS showed a tendency to be better in patients who received a combination of fluorouracil, leucovorin and oxaliplatin (FLO) than that of patients who received a combination of fluorouracil, leucovorin and cisplatin (FLP) (5.8 mo *vs* 3.9 mo, $P = 0.077$). On the other hand, the median OS time did not differ significantly (10.7 mo *vs* 8.8 mo, $P > 0.05$) between the two groups. Thereafter, the authors performed a post hoc subgroup analysis in patients older than 65 years, and the FLO regimen produced a significantly superior RR (41.3% *vs* 16.7%), median PFS (6.0 mo *vs* 3.1 mo, $P = 0.029$) and time-to-treatment failure (5.4 mo *vs* 2.4 mo, $P < 0.001$), and an improved median OS (13.9 mo *vs* 7.2 mo, $P = 0.08$) compared with the FLP regimen. In addition, there was significantly less toxicity with FLO in this trial.

The comparison of irinotecan-containing versus non-irinotecan-containing regimens (mainly fluorouracil-cisplatin) showed a nonstatistically significant trend toward better survival with irinotecan (HR for death: 0.86, 95%CI: 0.73-1.02) in the previous meta-analysis[5]. Furthermore, irinotecan-based regimens have also been tested comprehensively and found to be active in single arm and randomized clinical trials[29-34]. In a phase Ⅲ randomized trial performed by Dank *et al*[32], irinotecan in combination with fluorouracil and folinic acid (IF) was compared with the combination of cisplatin and infusional fluorouracil (CF) in patients with advanced adenocarcinoma of esophagogastric cancer. The results of this trial showed that the IF regimen resulted in improved TTP, but not OS, compared with CF. However, IF was better with respect to toxic deaths, discontinuation for toxicity, severe neutropenia, thrombocytopenia and stomatitis. The authors concluded that IF may provide an acceptable, platinum-free front-line treatment alternative for metastatic gastric cancer. Another phase Ⅱ trial revealed that the combination of capecitabine and irinotecan had a similar ORR (37.7% *vs* 42%, respectively) and median PFS (4.2 mo *vs* 4.8 mo, respectively), but a trend towards better median OS (10.2 mo *vs* 7.9 mo, respectively) than the capecitabine-cisplatin regimen[34].

S-1 is an oral fluoropyrimidine that includes three different agents: tegafur, gimeracil (5-chloro-2,4 dihydropyridine) and oteracil (potassium oxonate). This novel oral agent has shown promising results in patients with advanced gastric cancer, but the majority of data supporting the use of S-1 for advanced gastric cancer are from studies including Asian patients[5,35]. The randomized phase Ⅲ SPIRITS trial in 298 patients with advanced gastric cancer showed that both the median PFS (6.0 mo *vs* 4.0 mo) and median OS (13 mo *vs* 11 mo, $P = 0.04$) for patients who received combined S1 plus cisplatin were significantly better than those of patients who received S-1 alone in an Asian population. On the other hand, the grade 3 and 4 toxicity rates were significantly higher[36].

Tegafur is metabolized differently in Western and Asian populations, and as a result, the maximally tolerated dose also differs. Therefore, Western experience with combined S-1 plus cisplatin for advanced gastric cancer is limited, but also promising[37,38]. In their phase Ⅲ FLAGS trial including 1053 patients with advanced esophagogastric adenocarcinoma, Ajani *et al*[39] randomized patients to cisplatin plus either 5-FU or S-1. They showed that the median OS was not significantly inferior with S-1/cisplatin compared to the CF regimen (8.6 mo *vs* 7.9 mo). In addition, S-1/cisplatin was associated with a more favorable side effect profile and fewer treatment-related deaths[39]. It is thought that the lower cisplatin dose intensity in the S-1/cisplatin arm (75 mg/m^2 *vs* 100 mg/m^2) may have contributed to the survival and toxicity results. Despite the results of the FLAGS trial, future studies are needed to confirm the activity of S-1 in Western populations. Recently, updated results of the phase Ⅲ START trial presented at the 2012 ESMO meeting showed that among the 635 patients with metastatic gastric cancer analysed, the median OS time was 12.48 mo when S-1 was combined with docetaxel compared to 10.78 mo in patients who received S-1 alone. Neutropenia was the most

Table 1 Selected phase III clinical trials of current chemotherapy regimens for patients with advanced gastric cancer in the first-line setting

Ref.	Regimen	No. of patients	Response rate	Median PFS/TTP and OS (mo)
Van Cutsem *et al*[23]	DCF *vs* CF	445	37% *vs* 25%	TTP, 5.6 *vs* 3.7; OS, 9.2 *vs* 8.6
Cunningham *et al*[20]	EOF *vs* EOX *vs* ECX *vs* ECF	1002	42.4% *vs* 47.9% *vs* 46.4% *vs* 40.7%	PFS, 6.5 *vs* 7.0 *vs* 6.7 *vs* 6.2; OS, 9.3 *vs* 11.2 *vs* 9.9 *vs* 9.9
Kang *et al*[21]	CX *vs* CF	316	41% *vs* 29%	PFS, 5.6 *vs* 5.0; OS, 10.5 *vs* 9.3
Al-Batran *et al*[24]	FLC *vs* FLO	220	34.8% *vs* 24.5%	PFS, 5.8 *vs* 3.9; OS, 10.7 *vs* 8.8
Dank *et al*[32]	IF *vs* CF	333	31.8% *vs* 25.8%	TTP, 5.0 *vs* 4.2; OS, 9.0 *vs* 8.7
Koizumi *et al*[36]	CS *vs* S	305	54% *vs* 31%	PFS, 6.0 *vs* 4.0; OS, 13 *vs* 11
Ajani *et al*[39]	CS *vs* CF	1053	29.1% *vs* 31.9%	PFS, 4.8 *vs* 5.5; OS, 8.6 *vs* 7.9
Yoshida *et al*[40]	DS *vs* S	635	38.8% *vs* 26.8%	PFS, 5.29 *vs* 4.17; OS, 12.48 *vs* 10.78

DCF: Docetaxel, cisplatin and fluorouracil; CF: Cisplatin and fluorouracil; EOF: Epirubicin, oxaliplatin and fluorouracil; EOX: Epirubicin, oxaliplatin and capecitabine; ECX: Epirubicin, cisplatin and capecitabine; ECF: Epirubicin, cisplatin and fluorouracil; CX: Cisplatin and capecitabine; FLO: Fluorouracil, leucovorin and oxaliplatin; FLC: Fluorouracil, leucovorin and cisplatin; IF: Irinotecan and cisplatin; CS: Cisplatin and S-1; S: S-1; DS: Docetaxel and S-1; PFS: Progression-free survival; OS: Overall survival; TTP: Time-to progression.

frequent adverse event in the docetaxel/S-1 arm, with one death occurring from grade 4 thrombocytopenia[40]. Selected phase Ⅲ clinical trials of current chemotherapy regimens for patients with advanced gastric cancer in the first-line setting are summarized in Table 1.

Targeted therapy

Anti-HER2 agents: EGFR overexpression has been found in different cancer types including gastric cancer and is believed to be associated with tumor invasion, high grade histology, and poor prognosis[41]. The EGFR family comprises four members, of which epidermal growth factor receptor 1 (EGFR1) and HER2 (EGFR-Ⅱ) have been comprehensively investigated as targets for drugs in patients with metastatic gastric cancer. HER2 amplification and HER2 overexpression increase from 12% to 27% and 9% to 23%, respectively, in esophagogastric cancer, a similar percentage to that seen in breast cancer[42-46]. HER2 positivity is reported to be more frequent in patients with intestinal histology (34%) than in those with diffuse-type histology (6%), as well as in gastroesophageal junction (32%) compared to gastric cancer (18%)[46].

The trastuzumab for gastric cancer (ToGA) trial, a pivotal randomized, prospective, multicenter, phase Ⅲ clinical trial, evaluated the efficacy of anti-HER2 trastuzumab in combination with chemotherapy in patients with HER-2-positive advanced, mostly metastatic, gastric cancer[8]. After screening 3807 patients, 584 eligible HER2-positive patients according to immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH), were randomized to trastuzumab plus chemotherapy (fluorouracil or capecitabine and cisplatin) or chemotherapy alone. The study treatment was administered every 3 wk for six cycles, and trastuzumab was continued every 3 wk until disease progression, unacceptable toxicity, or withdrawal of consent. Crossover to trastuzumab at disease progression was not permitted. The ORR was significantly higher in the trastuzumab-containing arm (47% *vs* 35%). At a median follow-up of 17.1 to 18.6 mo, the median OS was significantly longer in the trastuzumab-containing arm (13.8 mo *vs* 11.1 mo, HR = 0.74, 95%CI: 0.60-0.91, $P = 0.0046$). There was no significant difference in rates of any adverse event, and cardiotoxicity was equally rare in both arms (an asymptomatic decrease in the left ventricular ejection fraction, 5% *vs* 1%). Grade 3 to 4 heart failure was reported in one and two patients, respectively. In subgroup analysis, trastuzumab was most effective in prolonging survival in the subgroup of patients with IHC 3+ tumors (HR = 0.66, 95%CI: 0.50-0.87), less effective in patients with IHC 2+ tumors (HR = 0.78, 95%CI: 0.55-1.10), and ineffective in those with *HER2* gene-amplified, but non protein-expressing (IHC 0 or 1+) tumors[8]. In the light of these findings, trastuzumab in combination with chemotherapy was approved to be a new standard of care for patients with HER2-positive advanced esophagogastric adenocarcinoma. Therefore, all patients with metastatic gastroesophageal adenocarcinoma should be evaluated in terms of HER2 status.

Lapatinib, an orally active tyrosine kinase inhibitor, has double targeted inhibition of both EGFR1 and HER2. The results of the randomized, phase Ⅲ TyTAN trial were presented at the 2013 ASCO Gastrointestinal Cancer Symposium[47]. The addition of lapatinib produced no significant benefit with respect to PFS (5.4 mo *vs* 4.4 mo) or OS (11.0 mo *vs* 8.9 mo) in the intent to treat population of advanced gastric cancer. On the other hand, there was significant benefit in both PFS (5.6 mo *vs* 4.2 mo) and OS (14.0 mo *vs* 7.6 mo) for patients with IHC 3+. The preliminary results of the TRIO-013/LOGiC trial were presented at the 2013 ASCO annual meeting[48]. In 545 patients with advanced gastroesophageal cancer, the benefit derived from the addition of lapatinib to chemotherapy was tested in first-line treatment. The combination of lapatinib and capecitabine/oxaliplatin did not significantly improve the median OS (12.2 mo *vs* 10.5 mo, HR = 0.91, 95%CI: 0.73-1.12) compared with chemotherapy alone. No correlation between intensity of staining for HER2 by IHC and outcomes was found. However, in subgroup analysis, Asian patients (median OS, 16.5 mo *vs* 10.9 mo, HR 0.68) and those under age 60 (median OS, 12.9 mo *vs* 9 mo, HR 0.69) seemed to benefit from lapatinib. The addition of lapatinib was as-

sociated with increased toxicity.

Anti-EGFR1 agents: EGFR is a transmembrane tyrosine kinase receptor involved in the proliferation and survival of cancer cells. EGFR overexpression is associated with advanced stages and poor prognosis in gastric cancer patients[49], and EGFR expression has been reported in 60% of gastric cancer patients[50,51]. Anti-EGFR monoclonal antibodies bind to the extracellular domain of EGFR in its inactive state; they compete for receptor binding by occluding the ligand-binding region, and thereby block ligand-induced EGFR tyrosine kinase activation. Cetuximab is a chimeric monoclonal antibody targeting EGFR and it's inhibition prevents tumor cell growth, angiogenesis, invasion, and metastasis, and induces apoptosis. The efficacy of this anti-EGFR monoclonal antibody in combination with chemotherapy has been reported in several phase Ⅱ clinical trials[52-54]. On the other hand, the benefit derived from the addition of cetuximab to chemotherapy could not be confirmed in a phase Ⅲ trial comparing chemotherapy alone in the first-line setting. In a recent phase Ⅲ (EXPAND) trial, 904 patients with advanced gastroesophageal adenocarcinoma were randomized to capecitabine and cisplatin with or without cetuximab[55]. The median PFS for patients who received the cetuximab-chemotherapy regimen was 4.4 mo compared with 5.6 mo for patients treated with chemotherapy alone (HR = 1.09, 95%CI: 0.92-1.29, P = 0.32). Moreover, the cetuximab arm resulted in more grade 3-4 adverse events (88% *vs* 77%). Similar results were reported in another phase Ⅲ trial of panitumumab. The REAL3 trial evaluated the benefit of the addition of panitumumab to chemotherapy in 553 patients with previously untreated advanced unselected esophagogastric cancer[56]. Patients were randomly allocated (1:1) to receive up to eight 21-day cycles of EOX or modified EOX (with a reduction in oxaliplatin to 100 mg/m^2 and capecitabine to 1000 mg/m^2 per day) plus panitumumab. The authors indicated that the addition of panitumumab was associated with a similar response rate but a significantly worse OS (median 8.8 mo *vs* 11.3 mo). In the light of these results, the addition of an anti-EGFR antibody to chemotherapy cannot be considered a standard approach for patients with advanced esophagogastric adenocarcinoma.

Small molecule tyrosine kinase inhibitors (TKI) have also been tested for advanced esophagogastric cancer in phase Ⅱ trials. The activity of erlotinib was suggested in patients with unresectable or metastatic adenocarcinoma originating in the EGJ or stomach in first-line treatment in the SWOG trial[57]. Six of the 70 patients obtained an ORR (9%, one complete), all of whom had EGJ tumors. The predictive significance of EGFR expression with respect to clinical outcome was not shown.

Anti-VEGF/VEGFR agents: VEGF is overexpressed by up to 60% and its overexpression correlates with an advanced stage, higher risk of recurrence and tumor aggressiveness and is an indicator for poor prognosis[58-60]. Anti-VEGF agents have recently been developed and comprise monoclonal antibodies and TKIs.

Bevacizumab is a humanized monoclonal antibody against VEGF, which is an endothelial cell-specific mitogen and the most potent driver of angiogenesis in tumorigenesis as it increases microvascular permeability. The inhibition of VEGF-A prevents pathological angiogenesis by inhibiting its interaction with VEGFR-2. This inhibition by bevacizumab has had a positive impact on patient outcomes in several malignancies including colorectal, lung, and renal cell carcinoma, as well as recurrent glioblastoma[50]. Several phase Ⅱ trials produced promising results when using bevacizumab in combination with different chemotherapeutic agents in treatment-naive patients with locally advanced or metastatic gastric cancer[61-63].

The recently published AVAGAST, phase Ⅲ trial evaluated the benefit of bevasizumab in combination with cisplatin and capecitabine as a first-line therapy in 774 patients with advanced gastric carcinoma[64]. Patients received capecitabine and cisplatin (XP) in combination with either bevacizumab or a placebo. AVAGAST did not reach its primary endpoint with no significant difference in OS (12.1 mo in bevacizumab-arm *vs* 10.1 mo in placebo-chemotherapy arm; HR = 0.87, P = 0.1002); however, both PFS (6.7 mo *vs* 5.3 mo, HR = 0.80, P = 0.0037) and ORR (46.0% *vs* 37.4%, P = 0.0315) improved significantly in the bevacizumab arm. In an unplanned subgroup analysis, OS for the pan-American subgroup was 6.8 mo for placebo *vs* 11.5 mo for bevacizumab (HR = 0.63). For European and Asian-Pacific subgroups, the OS was 8.6 *vs* 11.1 mo (HR = 0.85), and 12.1 mo *vs* 13.9 mo (HR = 0.97), respectively, with all results favoring bevacizumab. It was not clear whether the discrepancy came from genetic differences in ethnicity or from differences in treatment patterns, but Asian patients had fewer EGJ primaries, a lower frequency of liver metastases, and received second-line chemotherapy more often than did pan-American patients. Similar negative results for the addition of bevacizumab to XP in Asian patients with advanced gastric cancer were also presented at the 2012 ASCO Gastrointestinal Cancers Symposium in a preliminary report of the AVATAR study[65].

Ramucirumab (IMC-1121B) is a fully humanized monoclonal antibody aganist VEGFR-2[66]. Several phase Ⅱ and phase Ⅲ trials are currently underway or planned including ramucirumab plus chemotherapy *vs* chemotherapy plus placebo or best supportive care in both the first- and second-line setting (NCT00917384, NCT01170663, NCT01246960).

Apatinib is a TKI agent targeting VEGFR-2 (VEGFR), and its anti-angiogenesis effect has been demonstrated in preclinical tests. A recently published phase Ⅱ trial tested apatinib in patients with chemotherapy-refractory advanced metastatic gastric cancer. The median OS times were 2.50, 4.83 and 4.27 mo, in the placebo, apatinib 850 mg, once and apatinib 450 mg, twice daily arms respectively, and the median PFS times were 1.40, 3.67, and 3.20

Table 2 Phase-Ⅲ trials regarding targeted therapies in advanced gastric cancer

Ref.	Study/setting	Treatment	No. of patients	Response rate	Median PFS/TTP and OS (mo)
Anti-HER2 agents					
Bang *et al*[8]	ToGA/first-line	Trastuzumab + CX/CF *vs* CX/CF	584	47% *vs* 35%	PFS, 6.7 *vs* 5.5; OS, 13.8 *vs* 11.1
Bang *et al*[47]	TyTAN/second-line	Lapatinib + P *vs* P	430	NA	PFS, 5.4 *vs* 4.4; OS, 11.0 *vs* 8.9
Hecht *et al*[48]	TRIO-013/LOGiC/first-line	Lapatinib + CAPOX *vs* CAPOX	545	53% *vs* 40%	PFS, 6.0 *vs* 5.4; OS, 12.2 *vs* 10.5
Anti EGFR1 agents					
Lordick *et al*[55]	EXPAND/first-line	Cetuximab + CX *vs* CX	904	29% *vs* 30%	PFS, 4.4 *vs* 5.6; OS, 9.4 *vs* 10.7
Waddell *et al*[56]	REAL-3/first-line	Panitumumab + mEOX *vs* EOX	553	42% *vs* 46%	PFS, 6.0 *vs* 7.4; OS, 8.8 *vs* 11.3
Anti-VEGF agents					
Ohtsu *et al*[64]	AVAGAST/first-line	Bevacizumab + CX *vs* placebo + CX	774	46% *vs* 37.4%	PFS, 6.7 *vs* 5.3; OS, 12.1 *vs* 10.1
mTOR inhibitors					
Ohtsu *et al*[76]	GRANITE-1/first-line	Everolimus + BSC *vs* placebo + BSC	656	4.5% *vs* 2.1%	PFS, 1.7 *vs* 1.4; OS, 5.4 *vs* 4.3

HER2: Human epidermal growth factor receptor 2; EGFR1: Epidermal growth factor receptor 1; VEGF: Vascular endothelial growth factor; mTOR: Mammalian target of rapamycin; CX: Cisplatin and capecitabine; CF: Cisplatin and fluorouracil; P: Paclitaxel; CAPOX: Capecitabine and oxaliplatin; EOX: Epirubicin, oxaliplatin and capecitabine; mEOX: Modified EOX; BSC: Best supportive care; PFS: Progression-free survival; TTP: Time-to progression; OS: Overall survival; NA: Not applicable.

mo respectively. The differences between the apatinib and placebo groups were statistically significant for both PFS ($P < 0.001$) and OS ($P < 0.001$ and 0.0017)[67]. Toxicities were tolerable or manageable. A phase Ⅲ trial evaluating apatinib *vs* placebo for patients with advanced gastric cancer in the third-line setting is ongoing (NCT01512745).

Sunitinib and sorafenib are multi-target TKIs that also inhibit the VEGF receptor, as well as other TKs. Early reported phase Ⅱ trials have indicated mixed results. In a phase Ⅱ trial of sunitinib monotherapy for second-line treatment of metastatic gastric cancer, a partial response was obtained in only two of 78 patients, while another 25 showed a best response of stable disease ⩾ 6 wk. Median PFS and OS were 2.3 and 6.8 mo, respectively[68]. Another open-label randomized phase Ⅱ trial for the second-line treatment of 107 patients with unresectable or metastatic gastric cancer evaluated the combination of sunitinib plus docetaxel *vs* docetaxel monotherapy[69]. Although the sunitinib arm was associated with a significantly higher ORR, there was no significant difference in either TTP or OS. The combination of sorafenib plus docetaxel and cisplatin was tested in a phase Ⅱ trial in the first-line setting for patients with locally advanced or metastatic esophagogastric adenocarcinoma[70]. This trial demonstrated that the ORR was 41% and the median OS was 13.6 mo; the major grade 3 or 4 toxicity was neutropenia. However, these results will need to be further evaluated in a randomized trial in comparison with historical data on docetaxel plus cisplatin alone. There are a number of studies of locally advanced or metastatic gastroesophageal adenocarcinoma patients currently underway or planned for sunitinib and sorafenib combined with capecitabine-cisplatin or oxaliplatin-capecitabine, S-1-cisplatin, the FOLFIRI regimen, and new agents (NCT00555620, NCT00524186, NCT01020630). Table 2 shows selected phase Ⅲ clinical trials of targeted therapies in patients with advanced gastric cancer.

Other targeted agents: Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that integrates multiple signals from growth factors and hormones and plays a central role in the control of cell survival, hyperplasia, apoptosis, and other important physiological functions critical to tumorigenesis and cancer development, and is thus a potential target of anti-cancer therapy[71]. The first mTOR targeting agent was everolimus, an oral mTOR serine/threonine kinase inhibitor approved for the treatment of renal cell carcinoma, breast cancer, and progressive neuroendocrine tumors of pancreatic origin[72-74]. A phase Ⅱ study performed by Doi *et al*[75], in 53 patients with previously treated metastatic gastric cancer, reported that the median PFS and OS times were 2.7 and 10.1 mo, respectively, with good tolerability. A subsequent phase Ⅲ GRANITE-1 trial evaluated everolimus or BSC plus placebo in 656 previously treated advanced gastric cancer patients and the results of this trial showed insignificant benefit for the median OS (5.39 mo) in the everolimus arm when compared to the placebo arm (4.3 mo, $P = 0.1244$). On the other hand, promising results regarding PFS with everolimus treatment were reported in this study. The median PFS time was 1.68 mo in patients who received everolimus compared with patients treated with placebo (1.41 mo, HR = 0.68, $P < 0.0001$)[76]. There are currently several ongoing phase Ⅱ and Ⅲ studies in metastatic gastroesophageal adenocarcinoma patients comparing everolimus combined with paclitaxel, 5-FU, cisplatin, leucovorin and capecitabine (NCT01248403, NCT00632268, NCT01099527).

c-MET (mesenchymal-epithelial transition factor) is an oncogene encoding membrane TK receptor, and binding of hepatocyte growth factor (HGF), its ligand, to the receptor TK MET is implicated in the malignant process of multiple cancers, making disruption of this interaction a promising therapeutic strategy. MET expression or amplification has been found to be associated with poor prognosis in gastric cancer[77,78]. Onartuzumab is a humanized, monovalent (one-armed) monoclonal antibody against the MET receptor and blocks HGF binding to MET. The efficacy and safety of onartuzumab in combination with mFOLFOX6 in pa-

tients with metastatic HER2-negative gastroesophageal adenocarcinoma is currently being evaluated in phase Ⅱ and Ⅲ trials (NCT01590719, NCT01662869). Rilotumumab is another human monoclonal antibody (IgG2) against HGF that blocks binding of HGF to its receptor MET, inhibiting HGF/MET-driven activities in cells. A phase Ⅱ, double-blind, randomized study evaluated the efficacy and safety of rilotumumab with ECX regimen in patients with previously untreated metastatic gastroesophageal adenocarcinoma. The primary results of this study showed that the primary endpoint of PFS showed a tendency for a better outcome with rilotumumab plus ECX. The addition of rilotumumab to chemotherapy improved the median PFS from 4.2 to 5.6 mo (HR = 0.64). The secondary endpoint of OS also trended in favor of rilotumumab, with an improved median OS from 8.9 to 11.1 mo (HR = 0.73). The most common adverse events seen in the rilotumumab plus ECX arms were peripheral edema, neutropenia, anemia, thrombocytopenia and deep vein thrombosis. An exploratory analysis according to the MET protein expression level was presented at the 2012 ASCO annual meeting[79]. The addition of rilotumumab to ECX chemotherapy in patients with gastric tumors with high MET expression improved the median OS from 5.7 to 11.1 mo (HR = 0.29, P = 0.012). Conversely, in patients with gastric tumors with low MET expression, the addition of rilotumumab to chemotherapy was associated with a trend towards unfavorable OS (HR = 1.84). These results have led to a phase Ⅲ study to confirm the efficacy of rilotumumab in advanced esophagogastric cancer with high MET expression. This study is currently ongoing (RILOMET-1 trial, NCT01697072).

According to pre-clinical studies, histone deacetylase inhibitors (HDAC) have been found to be potential therapeutic targets in gastric cancer[80]. Vorinostat is a novel targeted agent that prevents tumor cell proliferation, survival and angiogenesis through histone deacetylase inhibition. Phase Ⅰ/Ⅱ studies comparing the effect of vorinostat with that of standard chemotherapy regimens in patients with advanced gastric cancer are underway (NCT01045538 and NCT00537121).

Second-line chemotherapy

Despite the improvement in survival of patients with metastatic gastric cancer, most patients develop progression of disease after first-line chemotherapy. Some patients with gastric cancer after failure of the first-line regimen are treated with second-line chemotherapy, but there was no standard second-line option until the positive results of recent phase Ⅲ trials[81]. In a Korean trial 202 patients with advanced gastric cancer who had received one or two prior chemotherapy regimens involving both a fluoropyrimidine and a platinum agent, and with a performance status (PS) of 0 or 1, were randomly assigned to either salvage chemotherapy (docetaxel 60 mg/m^2 every 3 wk or irinotecan 150 mg/m^2 every 2 wk) or best supportive care in a 2:1 fashion[82]. The authors showed that second-line chemotherapy was associated with a significant improvement in median OS (5.3 mo) versus BSC (3.8 mo) (HR = 0.657, P = 0.007), and patients were also significantly more likely to receive further salvage chemotherapy. There was no difference in median OS between docetaxel and irinotecan (5.2 mo *vs* 6.5 mo, P = 0.116).

In a smaller randomized, AIO trial carried out by Thuss-Patience *et al*[83], 40 patients with tumor progression after first-line chemotherapy and a PS of 0-2 were randomized to BSC or single-agent irinotecan. The median OS was significantly longer for patients treated with irinotecan chemotherapy than that of patients who received BSC (4 mo *vs* 2.4 mo, HR = 0.48, P = 0.012).

Similarly, the phase Ⅲ COUGAR-02 trial showed a modest survival benefit for single-agent docetaxel (75 mg/m^2 every 3 wk) in 168 patients who progressed within 6 mo of a platinum/fluoropyrimidine chemotherapy regimen. A preliminary report of this trial was presented at the 2013 ASCO annual meeting and the addition of docetaxel to BSC was associated with few ORR (7%), stable disease in 46% and a modest but statistically significant prolongation of median OS (5.2 mo *vs* 3.6 mo)[84]. A high rate of grade 4 toxicity was noted in the docetaxel arm, but symptom scores for pain were significantly better.

A meta-analysis of these trials was recently published[81]. The authors indicated that a significant reduction in the risk of death (HR = 0.64, P < 0.0001) was found with second-line chemotherapy. In addition, subgroup analysis showed a significant reduction in the risk of death with both irinotecan (HR = 0.55, P = 0.0004) and docetaxel (HR = 0.71, P = 0.004). In conclusion, the authors reported evidence to support the efficacy of second-line chemotherapy in the treatment of metastatic gastric cancer. In the light of these findings, although not all patients may be eligible for second-line therapy, it should be considered an option in appropriate patients.

A results of randomized, phase Ⅲ, TCOG GI-0801 trial was presented at the 2013 ASCO Gastrointestinal Cancers Symposium and median PFS for irinotecan plus cisplatin (4.17 mo) was significantly better than irinotecan alone (3.03 mo; P = 0.0324) in patients with previously treated with S-1-based chemotherapy for advanced gastric cancer[85]. No significant differences were detected in the TTF and RR (TTF, 3.4 mo *vs* 2.9 mo; RR; 21.9% *vs* 16.4% with irinotecan plus cisplatin and irinotecan alone, respectively). OS was immature. Related adverse events were comparable with irinotecan plus cisplatin and irinotecan. The authors concluded that irinotecan in combination with cisplatin has promising efficacy for the second-line chemotherapy compared with single agent irinotecan for metastatic gastric cancer. Recent phase Ⅲ clinical trials of second-line chemotherapy regimens for patients with advanced gastric cancer after failure of the first-line regimen are described in Table 3.

CONCLUSION

Recent trials of multiple agent chemotherapy regimens have demonstrated positive results in terms of improved survival; however, the prognosis of patients with meta-

Table 3 Second-line chemotherapy trials in patients with advanced gastric cancer

Ref.	Regimen	No. of patients	Response rate	Median PFS/TTP and OS (mo)
Kang *et al*[82]	Docetaxel or irinotecan + BSC *vs* BSC	202	-	TTP, NA; OS, 5.3 *vs* 3.8
Thuss-Patience *et al*[83]	Irinotecan *vs* BSC	40	44% *vs* 5%	PFS, 2.6 *vs* NA; OS, 4.0 *vs* 2.4
Ford *et al*[84]	Docetaxel + BSC *vs* BSC	168	7% *vs* NA	PFS, 5.6 *vs* 5.0; OS, 5.2 *vs* 3.6
Shimada *et al*[85]	Irinotecan + cisplatin *vs* irinotecan	130	21.9% *vs* 16.4%	PFS, 4.17 *vs* 3.03; OS, NA

BSC: Best supportive care; NA: Not applicable; PFS: Progression-free survival; OS: Overall survival; TTP: Time-to progression.

static gastric cancer remains poor and responses to first-line chemotherapy are modest and heterogeneous. Therefore, in patients with refractory gastric cancer, although not all patients may be eligible, second-line chemotherapy should be considered an option in appropriate patients in the light of recent phase Ⅲ trials and meta-analyses. In order to improve the results of currently available treatments, clinical investigations of targeted agents have recently been conducted. Agents targeting EGFR1 and HER2 have been widely tested. The addition of trastuzumab to cisplatin/fluoropyrimidine-based chemotherapy significantly improved survival in patients with HER2-positive metastatic gastric cancer, which is now the new standard of care by recent ToGA trial. However, this benefit is limited to only approximately 20% of patients with metastatic gastric cancer. Therefore, there remains a critical need for both the development of more effective agents. Other clinical trials of agents targeting VEGF, mTOR, and other biological pathways, have shown marginally positive results. However, future studies are needed to confirm the benefit of adding these targeted agents to chemotherapy and for the detection of novel, molecular, predictive factors and therapeutic targets in order to identify better and optimal treatment modalities for metastatic gastric cancer.

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TOPIC HIGHLIGHT

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DNA methylation in gastric cancer, related to *Helicobacter pylori* and Epstein-Barr virus

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Abstract

Gastric cancer is a leading cause of cancer death worldwide, and significant effort has been focused on clarifying the pathology of gastric cancer. In particular, the development of genome-wide analysis tools has enabled the detection of genetic and epigenetic alterations in gastric cancer; for example, aberrant DNA methylation in gene promoter regions is thought to play a crucial role in gastric carcinogenesis. The etiological viewpoint is also essential for the study of gastric cancers, and two distinct pathogens, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are known to participate in gastric carcinogenesis. Chronic inflammation of the gastric epithelium due to *H. pylori* infection induces aberrant polyclonal methylation that may lead to an increased risk of gastric cancer. In addition, EBV infection is known to cause extensive methylation, and EBV-positive gastric cancers display a high methylation epigenotype, in which aberrant methylation extends to not only Polycomb repressive complex (PRC)-target genes in embryonic stem cells but also non-PRC-target genes. Here, we review aberrant DNA methylation in gastric cancer and the association between methylation and infection with *H. pylori* and EBV.

Key words: Gastric cancer; Epigenetics; DNA methylation; Epstein-Barr virus; *Helicobacter pylori*

Core tip: Recent technological advances in genome-wide analysis tools have revealed various molecular aberrations in cancer. Although gastric cancer involves multiple genetic and epigenetic alterations, aberrant DNA methylation in gene promoter regions is thought to play a critical role in gastric carcinogenesis. From the etiological viewpoint, two pathogens, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are known to participate in gastric carcinogenesis. Chronic inflammation in the gastric mucosa due to *H. pylori* and EBV infection of gastric epithelial cells has been reported to cause aberrant promoter methylation, which may contribute to the tumorigenic mechanisms of these pathogens.

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INTRODUCTION

Gastric cancer is a leading cause of cancer death worldwide[1]. Malignant tumors, including gastric cancer, are

known to arise through multiple genetic and epigenetic alterations[2], and these molecular changes eventually impact the expression of cancer-associated genes, such as oncogenes and tumor-suppressor genes. Historically, one of the most common genetic alterations in cancer is mutation of the *TP53* gene[3,4]. *TP53* is a core tumor-suppressor gene, and more than half of all gastric cancers demonstrate loss of *TP53* function due to genetic alterations[5]. Another example is *CDH1*, the gene encoding a calcium-dependent cell-to-cell adhesion glycoprotein that is responsible for familial diffuse type gastric cancers due to germline mutations[6]. However, sporadic gastric cancers also display *CDH1* somatic mutations at a constant rate[7]. Moreover, recent whole-genome exome analyses in gastric cancer have identified mutations in several genes, including *ARID1A*, *PIK3CA*, and *FAT4*[8,9].

Although gastric cancer involves various molecular alterations, aberrant promoter methylation plays a major role in gastric carcinogenesis[10-15]. $p16^{INK4A}$ is the most well-known tumor-suppressor gene that is silenced by promoter methylation; the promoter region of $p16^{INK4A}$ is aberrantly methylated in 25%-42% of gastric cancers[10,11,16,17], while mutations or deletions are very rare[16]. *RUNX3* is also a significant tumor-suppressor gene in gastric cancer[18], and approximately half of all gastric cancer cases lose *RUNX3* expression due to hemizygous deletion and promoter hypermethylation, while point mutations are rarely reported. Although mutations in DNA mismatch-repair genes such as *MLH1* and *MSH2* are quite rare in gastric cancers[19,20], promoter methylation of *MLH1* represents a major cause of microsatellite instability (MSI)[21,22], which is observed in 31%-67% of gastric cancers[19,23].

Several scanning methods have been developed to identify novel tumor-suppressor genes silenced by promoter methylation[24-30], and genome-wide analysis has demonstrated unusual clustering of aberrant methylation in a subset of cancer cases. The phenotype presenting atypical methylation of cytosine-phosphate-guanine (CpG) islands, termed the CpG island methylator phenotype (CIMP), was first described in colorectal cancers[31]. Gastric cancer was also evaluated using methylation markers for colorectal cancer CIMP, and CIMP was also found to be present in gastric cancer[10]. Genome-wide analysis of aberrant DNA methylation in gastric cancer was first performed using the methylation-sensitive-representational difference analysis (MS-RDA) method to identify methylation-associated silenced genes, including novel tumor-suppressor genes[32-34]. Using silenced genes as markers, a subset of gastric cancers was demonstrated to harbor unusual accumulation of aberrant methylation in promoter CpG islands[32].

Environmental factors are also significantly related to the induction of aberrant DNA methylation, and etiological studies have provided evidence that two distinct infectious agents, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), are closely associated with gastric carcinogenesis[35-37]. Here, we review aberrant DNA methylation in gastric cancer and the association between methylation and infection with these two unique pathogens.

EPIGENETIC MODIFICATION AND DNA METHYLATION

Physiological function of DNA methylation

"Epigenetics", as compared to "genetics", is defined as the study of genomic DNA modifications that are heritable during cell division but do not involve a change in the DNA sequence itself, such as DNA methylation and histone modification[12,13,38]. DNA methylation is the covalent modification of a methyl group on the 5-position of cytosine at CpG dinucleotides[38,39]. CpG islands are genomic regions that contain dense CpG dinucleotides, and they are located in the promoter regions of approximately half of all genes. CpG islands are generally free from DNA methylation, allowing for the expression of downstream genes whose transcription is regulated by histone modification[13].

In normal cellular processes, DNA methylation is used for robust gene silencing, such as genome imprinting[40] and X-chromosome inactivation[41]. Moreover, tissue-specific patterns of methylation or changes in methylation during cellular differentiation have been discovered at CpG-poor promoters[42] and inter- and intragenic CpG islands[43]. In addition, when cells encounter foreign nucleic acid, such as viral DNA, host cells take advantage of DNA methylation as a defensive system to inactivate foreign nucleic acid[44].

Aberrant DNA methylation in cancers

Broadly speaking, aberrant DNA methylation in cancer is divided into two categories: "genome-overall hypomethylation" and "regional hypermethylation". The former, global hypomethylation, was discovered in the 1980s[45] and can be defined as a decrease in 5-methylcytosine content throughout the genome. CpG dinucleotides show heterogeneous distribution, especially in repetitive sequences, which are typically methylated in normal tissue[46,47]. In cancers, these repetitive sequences demonstrate aberrant hypomethylation[48], promoting genomic instability and cancer progression[49-51]. Loss of imprinting is another example of an epigenetic alteration related to aberrant hypomethylation[52], and loss of imprinting in *IGF2* was shown to be involved in the early events of carcinogenesis and was associated with increased colorectal cancer risk[53,54]. A subset of male germ line-specific genes, specifically the *MAGE* gene families, was discovered to be a cancer antigen in malignant melanoma[55]. These genes are repressed by promoter methylation in normal somatic tissues but are activated through promoter hypomethylation in several types of cancers[56,57].

The latter type of DNA methylation, regional hypermethylation, arises in CpG islands[58-60]. Aberrant methylation of promoter CpG islands leads to inappropriate transcriptional silencing, and this phenomenon is

regarded as one of the major mechanisms for inactivating tumor-suppressor genes[2,12-14]. Promoter methylation in tumor-suppressor genes has been discovered in various cancers, including *RB* in sporadic retinoblastoma[61], *VHL* in renal cell carcinoma[62], *CDH1* in hepatocellular carcinoma[63], and $p16^{INK4A}$ in various cancers[64].

In embryonic stem (ES) cells, the Polycomb repressive complex (PRC) plays a significant role in reversibly repressing gene expression. In ES cells, these PRC-target genes are frequently methylated compared to non-PRC-target genes in various cancers[65]. Our comprehensive methylation analysis of gastric cancer revealed significant enrichment of aberrant methylation in PRC-target genes in a subset of gastric cancers with a high-methylation epigenotype[37]. However, another subset of gastric cancer demonstrated an extensively high methylation epigenotype that displayed extended methylation in both PRC-target genes and non-PRC-target genes. This phenotype was detected in EBV-positive gastric cancer, and it will be discussed in detail later in this review.

Among the factors known to cause aberrant DNA methylation in non-cancerous tissues, aging is known to promote the accumulation of DNA methylation[66,67]. Indeed, age-dependent promoter methylation could explain the association between cancer and aging[68]. Recent whole-genome bisulfite sequencing comparing newborn and centenarian genomes demonstrated that centenarian DNA had a lower DNA methylation content throughout the genome and showed the more hypomethylated CpGs in promoters, exonic, intronic, and intergenic regions, whereas a greater level of DNA methylation was observed in CpG island promoters[69]. Another report showed that replicative senescent human cells exhibited features similar to the cancer epigenome, such as widespread DNA hypomethylation and focal hypermethylation[70]. Epidemiological studies have also revealed that the epigenetic status is influenced by various environmental factors[67] and can be associated with cancer incidence or prognosis[71,72].

Among environmental factors, chronic inflammation is a significant inducer of aberrant DNA methylation, as demonstrated by the analysis of non-cancerous tissues, such as colonic mucosae with ulcerative colitis[73], liver tissue with chronic hepatitis[74], esophageal mucosae with inflammatory reflux esophagitis[75], and gastric mucosae with chronic gastritis[76]. In a mouse colitis model induced by dextran sodium sulfate, aberrant CpG island methylation in colonic epithelial cells was shown to accumulate gradually on a monthly basis[77]. Interestingly, even in severe combined immunodeficiency (SCID) mice lacking functional T and B lymphocytes, DNA methylation was induced at the same level as in the background strain of mice, suggesting that functional T and B lymphocytes are not essential for methylation accumulation.

H. PYLORI AND ABERRANT DNA METHYLATION

Two distinct pathogens, *H. pylori* and EBV, are known to be involved in gastric carcinogenesis. First, we will discuss the association between chronic inflammation due to *H. pylori* and DNA methylation.

H. pylori, discovered in 1983 by Marshall BJ and Warren JR[78], is a helix-shaped Gram-negative bacterium present in the stomach of approximately half of the world's population[79,80]. Recent prospective cohort studies indicate that *H. pylori* infection plays an essential role in various disorders, including gastric cancer[35,36], chronic gastritis[78], intestinal metaplasia[81,82], and gastric lymphoma[83]. In 1994, the World Health Organization concluded that "*H. pylori* is a definite carcinogen" based on epidemiological evidence[79,80].

Two pathways have been proposed to play a role in gastric carcinogenesis resulting from *H. pylori* infection: the direct interaction of *H. pylori* with the gastric epithelium and its indirect involvement through chronic inflammation. Ultimately, however, both of these pathways cooperate to promote gastric carcinogenesis.

Direct interaction of H. pylori with gastric epithelium

The direct mechanism by which *H. pylori* contributes to gastric carcinogenesis is attributed to its pathogenicity. Most Gram-negative bacteria exert pathogenicity through the acquisition of an exogenous gene cluster, termed a pathogenicity island (PAI). *H. pylori* also contains a *cag* PAI, which consists of an approximate 40-kbp stretch of DNA encoding approximately 30 genes, including those of the type Ⅳ secretion system[84]. The pathogenicity of *H. pylori* depends on whether it contains cytotoxin-associated gene A (CagA) protein or not. Almost all strains of *H. pylori* in East Asia contain the CagA protein, whereas this frequency in Western strains is limited to 60%. The pathogenicity of the CagA protein is exerted via injection into gastric epithelial cells through type Ⅳ secretion systems (Figure 1). The CagA protein contains a conserved motif in the C-terminus, the EPIYA motif, which dictates the severity of its pathogenicity. East Asian strains of CagA exert more aggressive cytotoxicity compared to Western strains[85].

Host cellular responses against injected CagA protein display several patterns. These include (1) enhanced cell motility that induces a growth-factor-like phenotype, termed hummingbird, in host gastric cells[86]; (2) disruption of the epithelial apical-junctional complex[87]; and (3) epithelial proliferative and proinflammatory responses associated with the development of chronic gastritis and gastric cancer[88]. Therefore, CagA plays a key role in gastric carcinogenesis, although the direct involvement of CagA or other components of *H. pylori* in the induction

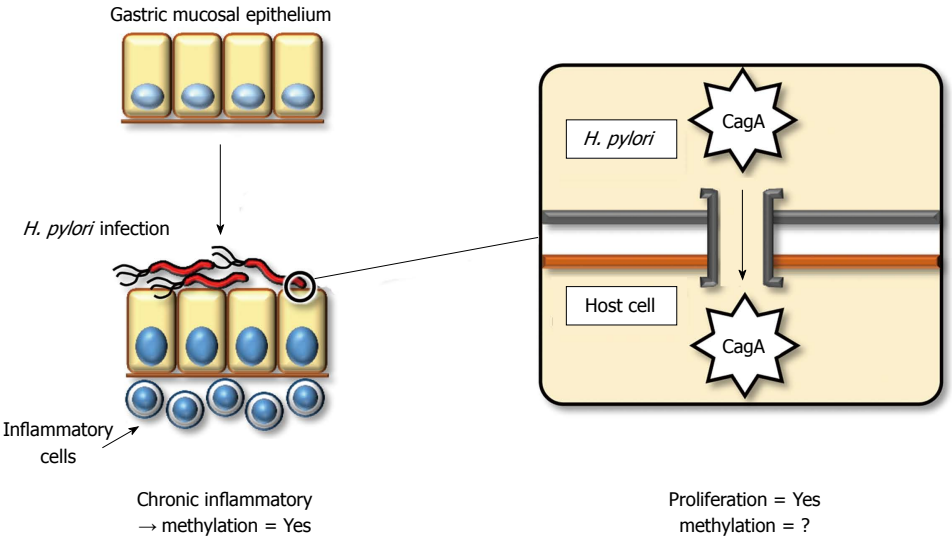

Figure 1 Schematic representation about infectious condition and pathogenicity of *Helicobacter pylori*. Pathogenicity of *Helicobacter pylori* (*H. pylori*) is exerted through injection of CagA into gastric epithelial cells using type Ⅳ secretion system. Inflammatory cell infiltration due to *H. pylori* might be more significant factor in induction of aberrant DNA methylation (left). Injection of CagA leads to proliferation of epithelial cells, but it is still unclear whether it plays an important role in methylation induction in epithelial cells (right).

of DNA methylation has not been clarified.

Chronic inflammation induced by H. pylori

H. pylori indirectly promotes pathogenicity by inducing chronic inflammation. Chronic inflammation generally involves the accumulation of molecular damage through a variety of mechanisms, such as DNA damage by free radicals[89] or aberrant expression of activation-induced cytidine deaminase (AID)[90]. Moreover, chronic inflammation induces aberrant DNA methylation[74]. Rather than *H. pylori* infection itself, inflammatory cell infiltration by *H. pylori* might be a more significant factor for the induction of aberrant DNA methylation[91]. In a Mongolian gerbil model, suppression of aberrant DNA methylation by 5-aza-dC treatment reduced, but did not entirely prevent, the incidence of *H. pylori*-induced gastric cancers[92]. This result demonstrates that aberrant DNA methylation contributes to *H. pylori*-related gastric carcinogenesis, although some direct influences of *H. pylori*, without aberrant DNA methylation, may also be significant.

H. pylori infection induces aberrant promoter methylation in tumor-suppressor genes, including $p16^{INK4A}$, *LOX*, and *CDH1*[34,93]. Although eradication of *H. pylori* can reduce the level of promoter methylation, a certain amount of methylation remains[91,94]. This observation suggests that not only fully differentiated gastric epithelial cells but also stem/progenitor cells might acquire aberrant methylation. In human ulcerative colitis and hepatitis, increased expression of *IL-1β*, *IL-8*, *NOS2*, and *TNF* was observed[95-98], and these genes may represent a common factor associated with the induction of aberrant DNA methylation during chronic inflammation. In particular, *IL-1β* is thought to be significant, as a specific single-nucleotide polymorphism of *IL-1β* is associated with increased gastric cancer risk and increased incidence of *CDH1* promoter methylation in gastric cancers[99,100]. Furthermore, the role of *IL-1β* in *H. pylori*-induced gastric inflammation and DNA methylation was confirmed using IL-1 receptor type 1 knockout mice[101].

EBV AND ABERRANT DNA METHYLATION

EBV is another pathogen known to be involved in gastric carcinogenesis.

EBV and gastric carcinogenesis

EBV is a gamma-herpes virus consisting of a double-strand DNA genome approximately 170 kbp in length. EBV may cause infectious mononucleosis during initial infection, and more than 90% of adult individuals become EBV carriers[102], as this virus can be maintained asymptomatically in a latent form in memory B lymphocytes. However, EBV displays the characteristics of an oncogenic virus; indeed, it was initially discovered in human neoplastic cells, specifically a Burkitt's lymphoma cell line, in 1964[103]. Subsequently, EBV was associated with several types of malignant tumors, such as nasopharyngeal carcinoma[104], Hodgkin lymphoma[105], and opportunistic lymphoma in immunocompromised individuals[106,107]. Moreover, a subgroup of gastric cancer patients infected with EBV was discovered in 1990[108], and this unique subgroup is distributed throughout the world, without regional or racial deviation, at a rate of 7%-15%[109, 110].

EBV-positive (EBV^+) gastric cancers show distinct clinicopathological features. First, EBV^+ gastric cancers demonstrate EBV infection in almost all neoplastic cells of the tumor, which has been confirmed by *in situ* hy-

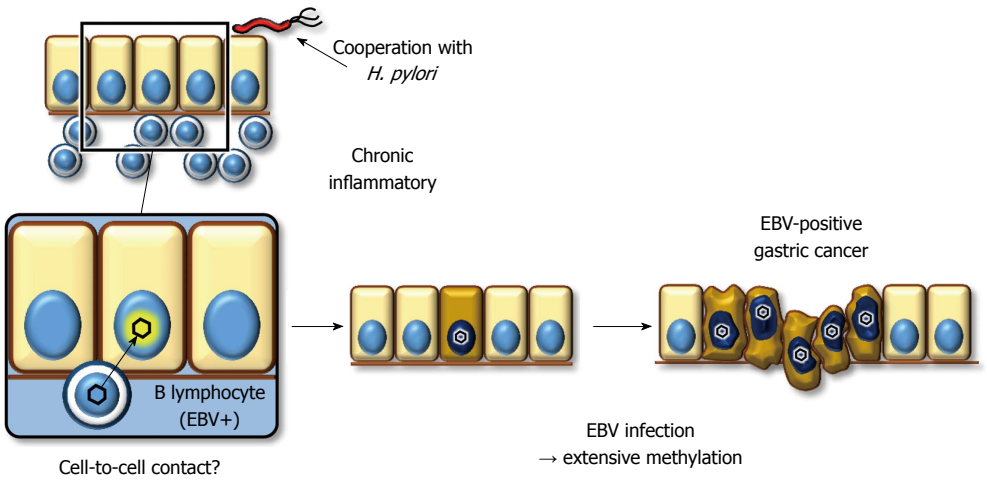

Figure 2 Schematic representation about infectious condition and pathogenicity of Epstein-Barr virus. Direct cell-to-cell contact between B lymphocyte and gastric epithelial cell may perhaps be the most likely model to infect with Epstein-Barr virus (EBV) into epithelial cells *in vivo* (left). EBV infection induces extensive promoter methylation, which should contribute to tumorigenesis. *H. pylori: Helicobacter pylori.*

bridization for a non-coding small RNA, EBER, which is abundantly expressed in the nuclei of infected neoplastic cells. In addition, the clinical features of EBV$^+$ gastric cancers differ from EBV-negative (EBV) gastric cancers due to their male predominance, proximal location, and relatively favorable prognosis[111]. Histopathologically EBV$^+$ gastric cancers demonstrate characteristic features of a poorly differentiated adenocarcinoma with marked infiltration of lymphocytes into the stromal tissue, which has been reported as "gastric cancer with lymphoid stroma"[112].

EBV has a double-stranded DNA genome that exists in a linear form in viral particles. After EBV enters the host cell, the viral DNA circularizes *via* the fusion of terminal repeats at both ends, and it maintains its circular form in the nuclei of latently infected cells without integration into the host genome[113]. Southern blot analysis for terminal repeats has demonstrated that EBV present in neoplastic cells is mono- or oligo-clonal, even in advanced stages[114-116]. Moreover, all of the cancerous cells are positive for EBER-*in situ* hybridization in all cases of EBV$^+$ gastric cancer. This fact indicates that EBV infection occurs at the initial, or a very early stage, of carcinoma development, and it implies a profound association of EBV with gastric carcinogenesis.

The mechanism underlying EBV infection in the gastric mucosal epithelium remains unclear, while the viral receptor molecule for CD21 in B lymphocytes is not expressed on epithelial cells[117]. Because co-cultivation of virus-producing lymphocytes demonstrates a much greater efficiency of infection (up to 800-fold) compared to cell-free infection, direct cell-to-cell contact between B lymphocytes and gastric epithelial cells is the most likely model to explain how EBV infects epithelial cells *in vivo* (Figure 2)[118]. This hypothesis supports histopathological data showing that the background mucosa of EBV$^+$ gastric cancer presents atrophic gastritis with lymphocyte infiltration due to *H. pylori* infection[119]. However, it remains unclear whether chronic inflammation with *H. pylori* is a prerequisite for EBV to infect gastric epithelial cells.

DNA methylation in EBV-positive gastric cancer

EBV$^+$ gastric cancer forms a distinct subgroup of gastric cancer. Previous reports have indicated that promoter methylation is observed more frequently in EBV$^+$ gastric cancers than in EBV gastric cancers, despite analyzing a limited number of cancer-associated genes[120-122]. We performed a comprehensive analysis of promoter methylation in clinical gastric cancers and found that gastric cancers clustered into three distinct subgroups. Interestingly, EBV$^+$ gastric cancers displayed an extremely high methylation phenotype, termed the EBV$^+$ epigenotype[37]. Moreover, genes specifically methylated in EBV$^+$ gastric cancers were shown to expand not only within PRC-target genes in ES cells but also to non-PRC-target genes. This result implies that EBV$^+$ gastric cancer is methylated *via* a unique mechanism(s). Subsequently, to clarify the causal role of EBV infection, we performed *in vitro* EBV infection experiments in low-methylation MKN7 gastric cancer cells to determine whether these cells would acquire extensive methylation and, as a result, the EBV$^+$-specific methylation epigenotype. The induced methylation repressed multiple genes, including multiple tumor-suppressor genes, suggesting a role for EBV in tumorigenesis[37].

The inducer of aberrant DNA methylation remains elusive. EBV exists in three latent forms defined by the expression pattern of latent genes. Lymphoblastoid cell line (LCL) and transformed primary B lymphocytes infected with EBV express all latent genes, LMPs (1, 2A, 2B), EBNAs (1, 2, 3A, 3B, 3C, LP), EBERs (1, 2), and BARTs, and this expression program is referred to as type Ⅲ latency. In contrast, Burkitt's lymphoma shows type I latency, with the minimum expression of EBNA1, EBERs, and BARTs only. Type Ⅱ latency, in which LMP1 and LMP2 are expressed in addition to

latency I genes, is observed in EBV-associated Hodgkin lymphoma, peripheral natural killer/T-cell lymphoma, and nasopharyngeal carcinoma. EBV$^+$ gastric cancer shows type I (or II) latency and expresses EBNA1, EBERs, BARTs, and LMP2A[105,111,123].

Several studies have elucidated the function of latent genes for promoter methylation. LMP1 was reported to down-regulate *CDH1* gene expression and induce cell migration using cellular DNA methylation machinery[124,125]. LMP2A plays an essential role in epigenetic abnormalities by inducing promoter methylation of *PTEN*[126]. EBER1 and EBER2 are small non-coding RNAs of approximately 170 bases in length that are abundantly expressed in the nuclei of latently infected cells, up to 10^7 copies per cell. Although some oncogenic properties of EBERs have been reported, such as the contribution of efficient growth transformation of B lymphocytes[127,128] or the induction of insulin-like growth factor 1 (IGF-I) acting as an autocrine growth factor in gastric cancer or nasopharyngeal carcinoma cells[129,130], the distinct influence of epigenetic modification remains unclear. Moreover, while these viral genes may play a role in aberrant methylation, methylation induction at the genome-wide scale, which can result from EBV infection, has not been demonstrated through the forced expression of any viral gene[37].

Rather than viral factors, host cellular mechanisms may play more important roles in the induction of aberrant methylation. In type I latency, while host cells induce dense methylation in the viral genome to silence most viral genes, the host genome itself is also extensively hypermethylated[131,132]. In type III latency, such as LCL, neither the viral genome nor the host cellular genome is significantly hypermethylated[132], and this observation implies that a host-driven mechanism that induces DNA methylation in the viral genome may affect methylation of the host genome. Recent exome sequencing analysis demonstrated that *ARID1A* was frequently mutated in EBV$^+$ gastric cancer[8,133], and other chromatin remodelers were also mutated[9]. While it is not known whether mutation of these genes is causally associated with aberrant methylation in EBV$^+$ gastric cancer, these chromatin remodelers may play a role in protecting the epigenomic status of the host genome from the pressure of methylation induction. Further investigation is necessary to clarify the roles of host cellular factors in methylation induction.

CONCLUSION

In gastric carcinogenesis, aberrant promoter methylation plays a major role by inactivating tumor-suppressor genes. Two pathogens, *H. pylori* and EBV, may contribute to carcinogenesis through the induction of aberrant methylation in gastric epithelial cells, although further study is necessary to elucidate the detailed molecular mechanisms underlying the induction of aberrant promoter methylation in response to infection with these two pathogens. Understanding these mechanisms could clarify the process of gastric carcinogenesis, and application of this knowledge for clinical use could aid in diagnosis, risk management, and prevention. Epigenetic aberrations can accumulate at early stages of carcinogenesis, preceding genomic mutations in polyclonal tissues; aberrant DNA methylation is therefore a powerful biomarker for the early detection of cancers and/or cancer risk. Moreover, from prophylactic or therapeutic viewpoints, aberrant DNA methylation could represent an attractive target due to its reversible nature. For example, a patient with persistent DNA methylation after *H. pylori* eradication might be a candidate for demethylation therapy to prevent gastric cancer. Moreover, in EBV$^+$ gastric cancers, aberrations in chromatin remodeling factors in background fields may promote EBV infection or carcinogenesis and could represent a target for the prevention of EBV$^+$ gastric cancer.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

An updated review of gastric cancer in the next-generation sequencing era: Insights from bench to bedside and *vice versa*

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Abstract

Gastric cancer (GC) is one of the most common malignancies and remains the second leading cause of cancer-related death worldwide. There is an increasing understanding of the roles that genetic and epigenetic alterations play in GCs. Recent studies using next-generation sequencing (NGS) have revealed a number of potential cancer-driving genes in GC. Whole-exome sequencing of GC has identified recurrent somatic mutations in the chromatin remodeling gene *ARID1A* and alterations in the cell adhesion gene FAT4, a member of the cadherin gene family. Mutations in chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) have been found in 47% of GCs. Whole-genome sequencing and whole-transcriptome sequencing analyses have also discovered novel alterations in GC. Recent studies of cancer epigenetics have revealed widespread alterations in genes involved in the epigenetic machinery, such as DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs and microRNAs. Recent advances in molecular research on GC have resulted in the introduction of new diagnostic and therapeutic strategies into clinical settings. The anti-human epidermal growth receptor 2 (HER2) antibody trastuzumab has led to an era of personalized therapy in GC. In addition, ramucirumab, a monoclonal antibody targeting vascular endothelial growth factor receptor (VEGFR)-2, is the first biological treatment that showed survival benefits as a single-agent therapy in patients with advanced GC who progressed after first-line chemotherapy. Using NGS to systematically identify gene alterations in GC is a promising approach with remarkable potential for investigating the pathogenesis of GC and identifying novel therapeutic targets, as well as useful biomarkers. In this review, we will summarize the recent advances in the understanding of the molecular pathogenesis of GC, focusing on the potential use of these genetic and epigenetic alterations as diagnostic biomarkers and novel therapeutic targets.

Key words: Next-generation sequencing; Microsatellite instability; MicroRNA; Epigenetic field defect; Gastric washes; Insulin-like growth factor 1 receptor

Core tip: The genetic and epigenetic alterations in gastric cancers (GC) have biological and clinical implications. Recent advances in the molecular research of GC have introduced new diagnostic and therapeutic strategies to clinical settings. In this review, we summarize the key findings of past reports pertaining to the genetics and epigenetics of GC and their relationship to and future applications in next-generation sequencing (NGS). We also describe the recurrently mutated genes and alterations in GC identified by NGS technology and discuss the basic framework for future investigations, including the challenges of using NGS as a tool for biomarker and therapeutic target discovery.

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INTRODUCTION

Gastric cancer (GC) is the second highest cause of global cancer mortality. GC is a heterogeneous disease with multiple environmental etiologies and alternative pathways of carcinogenesis[1,2]. One of the major etiologic risk factors for GC is *Helicobacter pylori* (*H. pylori*) infection, but only a small proportion of individuals infected with *H. pylori* develop GC[3,4]. There is an increasing understanding of the roles that genetic and epigenetic alterations play in GCs (Figure 1). Consequently, the development of appropriate biomarkers that reflect an individual's cancer risk is essential to reduce the mortality from GC[5,6]. Recent advances in molecular research of GC have brought new diagnostic and therapeutic strategies into clinical settings.

Next-generation sequencing (NGS) is a technology that involves the parallel sequencing of enormous amounts of short DNA strands from randomly fragmented copies of a genome[7,8]. NGS methods used for genome[9], exome[10], epigenome[11] and transcriptome[12] sequencing have the potential to provide novel avenues towards achieving a comprehensive understanding of diseases, including cancer[13,14]. Such advances have also shown puzzling tumor heterogeneity with limited somatic alterations shared between tumors of the same histopathologic subtype[15-17]. Although NGS techniques are just beginning to expand our abilities to detect genome-wide alterations in GC, several NGS studies in GC have recently been published[18].

In this review, we summarize the key findings of past reports pertaining to the genetics and epigenetics of GC and their relationship to and future application in NGS. We also describe the recurrently mutated genes and alterations in GC identified by NGS technology and discuss the basic framework for future investigations, including the challenges of using NGS as a tool for biomarker and therapeutic target discovery.

MICROSATELLITE INSTABILITY

A type of genetic instability characterized by alterations in length within simple repeat microsatellite sequences, termed microsatellite instability (MSI), occurs in approximately 15% of sporadic GCs, mainly as a result of epigenetic changes[19-22]. Genetic and epigenetic inactivation of DNA mismatch repair (MMR) genes leads to the mutator phenotype, mutations in cancer-related genes and cancer development (Figure 2). MSI underlies a distinctive carcinogenic pathway because MSI-positive (MSI^+) GCs exhibit many differences in clinical, pathological and molecular characteristics compared with MSI-negative (MSI^-) GCs[19-22]. The differences in genotype occur because defective MMR results in a strong mutator phenotype with a very specific mutation spectrum. MSI mainly accumulates frameshift mutations in the repeated sequences located in the coding regions of a target tumor suppressor or other tumor-related genes[23-26]. The atypical genotype of MSI^+ GCs also includes specific patterns of gene dysregulation. MSI^+ GCs often show epigenetic alterations, such as hypermethylation of various genes, including the key MMR gene *MLH1*. The differences in genotype and phenotype between MSI^+ and MSI^- GCs are likely linked to their differences in biological and clinical features. Recent findings from NGS analysis, such as the frequent mutation of the AT-rich interactive domain 1A (ARID1A) in MSI^+ GCs, support this notion[27,28].

The clinicopathological, genetic, epigenetic, prognostic and therapeutic characteristics of MSI^+ GCs are becoming clearer, but further research is still required. Because molecular targeting therapeutics are being used in clinical settings and trials, the differential regulation of molecular target genes in MSI^+ and MSI^- GCs[29,30] needs to be clarified. Diagnostic characterization of the MSI status of GCs thus has important implications for basic and clinical oncology.

Frequent inactivating mutations of ARID1A in molecular subtypes of GC identified by exome sequencing

Holbrook *et al*[31] analyzed 50 GC samples with targeted deep sequencing of the DNA of 384 genes. In addition to the previously reported mutations in genes belonging to various pathways, the authors found tractable target genes, such as the genes for the thyrotropin receptor and the Rho-associated coiled-coil containing protein kinases ROCK1 and ROCK2. Wang *et al*[27] performed exome

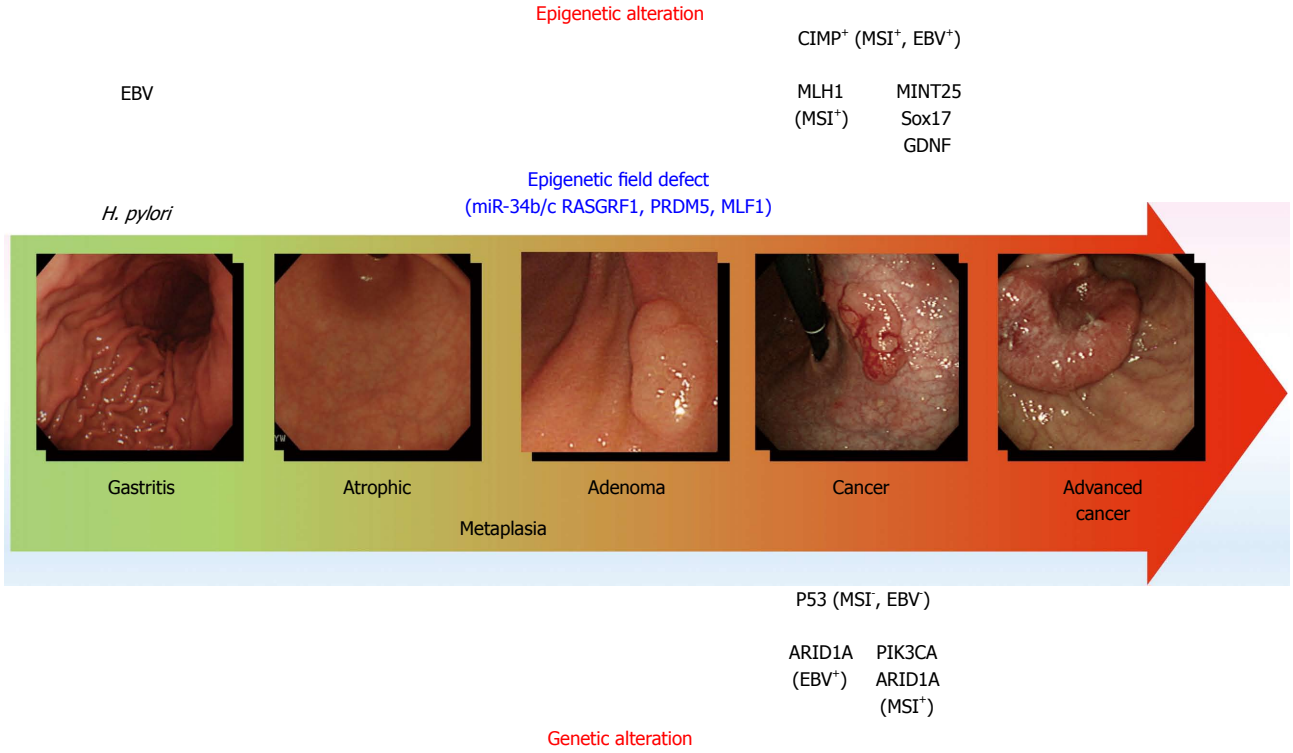

Figure 1 Genetic and epigenetic alterations in gastric carcinogenesis. The model for gastric carcinogenesis is presented based on genetic and epigenetic alterations. Methylation of the genes in blue appears to be involved in an epigenetic field defect. *H. pylori*: *Helicobacter pylori*; MSI: Microsatellite instability; EBV: Epstein-Barr virus; CIMP: CpG island methylator phenotype.

sequencing of 22 GC samples and found novel mutated genes and pathway alterations involved in chromatin modification. A validation study confirmed frequent inactivating mutations or protein loss of the ARID1A gene, which encodes one of the subunits in the Switch/Sucrose Nonfermentable (SWI-SNF) chromatin remodeling complex. The mutation spectrum for ARID1A differed among molecular subtypes of GC; mutations were detected in 83% of GCs with MSI, 73% of GCs with EBV infection and 11% of GCs without EBV and MSI. Moreover, ARID1A mutations were negatively associated with TP53 mutations. ARID1A alterations were associated with better prognosis in a stage-independent manner. These results suggest the importance of altered chromatin remodeling in the pathogenesis of GC.

Recurrent somatic mutations in cell adhesion and chromatin remodeling genes identified by exome sequencing

Zang *et al*[28] also analyzed a spectrum of somatic alterations in GC by sequencing the exomes of 15 GC specimens, including 11 intestinal-type, 1-mixed-type, and 3 diffuse-type adenocarcinomas and their matched normal DNAs. TP53 (11/15 tumors), PIK3CA (3/15) and ARID1A (3/15) were frequently mutated. Among the frequently mutated genes, cell adhesion was the most significant biological pathway affected. A prevalence screening confirmed mutations in FAT4, a member of the cadherin gene family, in 5% of GCs (6/110) and FAT4 genomic deletions in 4% (3/83) of GCs. Mutations in chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) were also found in 47% of GCs. ARID1A mutations were detected in 8% of GCs (9/110) and were associated with concurrent PIK3CA mutations and MSI. Both FAT4 and ARID1A showed tumor-suppressor activity in functional assays. Somatic inactivation of FAT4 and ARID1A may thus be key tumorigenic events in a subset of GCs. Because PI3K inhibitors are currently in clinical testing as treatment for GC[32], it will be interesting to evaluate whether the tumor responses to these compounds are affected by the genomic status of ARID1A.

Frequent loss of ARID1A expression in GC with EBV infection or MSI

Mutations of ARID1A lead to a loss of protein expression in GC and are particularly associated with EBV infection or MSI. Abe *et al*[33] investigated the significance of the loss of ARID1A in 857 GC cases, including 67 EBV^+ and 136 MLH1-lost MSI^+ GCs. Loss of ARID1A expression was significantly more frequent in cases of EBV^+ (23/67; 34%) and MSI^+ (40/136; 29%) GCs than in cases of EBV^-/MSI^- (32/657; 5%) GCs. Loss of ARID1A was correlated with larger tumor size, deeper depth of invasion, lymph node metastasis and poorer prognosis in cases of EBV^-/MSI^- GC. A correlation with tumor size and diffuse-type histology was found only in the MSI^+ GC; no correlation was observed in EBV^+ GC. Loss of ARID1A expression in EBV^+ GC was frequent in the early stage of GC, but EBV infection did not cause loss of ARID1A in GC cell lines. Thus, loss of ARID1A may be an early event in EBV^+ GC and may precede EBV infection in gastric epithelial cells. On the other hand,

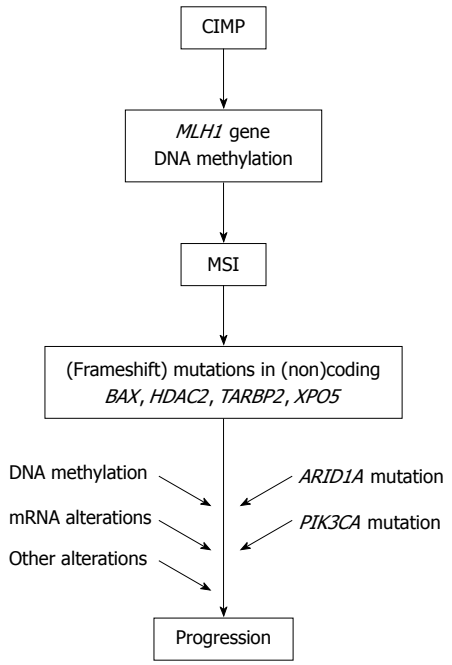

Figure 2 Molecular pathway for microsatellite instability+ gastric cancer. The model for the carcinogenesis of microsatellite instability (MSI)$^+$ gastric cancer is presented. CIMP: CpG island methylator phenotype.

loss of ARID1A may be involved in the progression of EBV/MSI GCs. Thus, loss of ARID1A appears to have different, pathway-dependent roles in GC.

WHOLE-GENOME SEQUENCING ANALYSIS OF GC

To explore the complete list of somatic alterations in GC, Nagarajan *et al*[34] combined massively parallel short read and DNA paired-end tag sequencing for the first whole-genome analysis of two GCs, one with CIN and the other with MSI. Integrative analysis and de novo assemblies revealed the architecture of a wild-type KRAS amplification, a common driver event in GC[35]. Three distinct mutational signatures were discovered against a genome-wide backdrop of oxidative and MSI-associated mutational signatures. Combining sequencing data from 40 complete GC exomes and targeted screening of an additional 94 independent GCs led to the discovery of ACVR2A, RPL22 and LMAN1 as recurrently mutated genes in MSI$^+$ GC and the identification of PAPPA as a recurrently mutated gene in TP53 wild-type GC. These results highlight how whole-genome sequencing analysis can provide relevant information about tissue-specific carcinogenesis that would otherwise be missed in exome-sequencing data. WGS of more GCs will uncover more recurrently altered genes.

miRNA alterations

A microRNA (miRNA) is a small noncoding RNA that regulates gene expression at the posttranscriptional level and is critical in many biological processes and cellular pathways[36-40]. The causes of aberrant miRNA expression patterns in cancer include DNA copy number amplification or deletion, inappropriate transactivation, transcriptional repression by oncogenic and other factors, failure of miRNA post-transcriptional regulation and genetic mutation or transcriptional silencing associated with hypermethylation of the CpG island promoters.

There is accumulating evidence to support the notion that miRNA alterations play a key role in the pathogenesis of GC[41-44]. A large number of miRNAs with different biological functions have been found to be altered and correlated with clinicopathological characteristics and/or prognosis in GC. Moreover, the clinical potential of miRNA alterations as minimally invasive diagnostic biomarkers and therapeutic targets has been extensively reported[37,40,42,44]. Recent studies have shown that tumor-derived miRNAs are present and stable in circulation, and the levels of circulating miRNAs are detectable and quantifiable. Both tissue and soluble miRNAs are candidates for diagnostic biomarkers and therapeutic targets in GCs[44]. The basic strategy of current miRNA-based treatment studies is to either antagonize the expression of target oncogenic miRNAs with antisense therapy and other technology or to restore the function of impaired tumor suppressor miRNAs[42].

The inclusion of different isoforms of miRNA (isomiRs) that are natural variants of mature miRNAs will form a detailed miRnome. Because expression of isomiRs can be estimated by NGS, NGS platforms provide the most effective method of miRNA profiling, leading to the identification of the miRNA alterations with clinical applications. Li *et al*[45] sequenced small RNAs from one pair of GC and noncancerous tissue and found that isomiR patterns are significantly different between these tissues. Moreover, these authors found that the 5p arm and 3p arm miRNAs derived from the same pre-miRNAs have different tissue preferences in GC and noncancerous tissue, suggesting a novel mechanism regulating mature miRNA selection.

WHOLE-TRANSCRIPTOME SEQUENCING OF GC

The first comprehensive RNA-seq study in GC has been recently published. Kim *et al*[46] applied a whole-transcriptome sequencing approach to 24 GC samples and six noncancerous tissue specimens. Importantly, these authors developed a multilayered integrative analysis to identify various types of transcriptional aberrations, such as differentially expressed mRNAs and miRNAs, as well as recurrently mutated genes. A central metabolic regulator gene, AMPKa2 (PRKAA2), was identified as a potential functional target in GC. Six key miRNAs (miR-548d-3p, miR-20b, miR-135b, miR-140-3p, miR-93 and miR-19a) in GC were also identified.

Epigenetic alterations

Epigenetic regulation is essential for the normal develop-

ment and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic regulation can lead to altered gene function and malignant cellular transformation[47]. Recent cancer epigenetic studies have revealed various alterations in the epigenetic machinery in GC, including DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs and miRNAs[48-52]. Aberrant DNA methylation in the promoter CpG islands of genes results in inactivation of tumor suppressor and other tumor-related genes in cancer cells and is the most well-defined epigenetic hallmark in GC. Methylation of a large number of genes with different biological functions has been found to be correlated with the clinicopathological characteristics and prognosis in GC[48-52]. DNA methylation with its advantages as a biomarker for the detection of cancer in biopsy specimens and body fluids that can be obtained non-invasively, such as serum and gastric washes, may have a clinical application in GC. Detection of aberrant DNA methylation of genes, such as *CDH1*, *DAPK*, *GSTP1*, *p15*, *p16*, *RARβ*, *RASSF1A*, *RUNX3* and *TFPI2*, in the serum may be a useful biomarker for the detection of GC[50]. Studies of DNA methylation and histone modification using NGS technologies, such as whole-genome bisulfite sequencing and targeted bisulfite sequencing, will lead to new discoveries and improve our knowledge of the epigenomics of GC[11].

Association of the aberrant methylation of RASGRF1 with an epigenetic field defect and an increased risk of GC

Aberrant DNA methylation is implicated in the epigenetic field defect seen in GC. Thus, it is important to identify predictive biomarkers by screening for DNA methylation in the noncancerous background gastric mucosa of patients with GC. Using methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis, Takamaru *et al*[53] found 224 genes that were methylated in the noncancerous gastric mucosa of patients with GC. Among them, RASGRF1 methylation was significantly elevated in the gastric mucosa from patients with either intestinal- or diffuse-type GC, compared with the mucosa from healthy individuals. RASGRF1 methylation was independent of mucosal atrophy and could be used to distinguish both serum pepsinogen test-positive and -negative patients with GC from healthy individuals. Ectopic expression of RASGRF1 suppressed colony formation and Matrigel invasion by GC cells. RASGRF1 methylation appears to be significantly involved in the epigenetic field defect of the stomach and to be a useful biomarker to identify individuals at high risk for GC.

Association of aberrant methylation of miR-34b/c with an epigenetic field defect and an increased risk of GC

The silencing of miRNAs is often associated with CpG island hypermethylation. Thus, to identify epigenetically silenced miRNAs in GC, Suzuki *et al*[54] screened for miRNAs that were induced by treatment of GC cells with 5-aza-2'-deoxycytidine and 4-phenylbutyrate. Hypermethylation of the neighboring CpG island epigenetically silenced miR-34b and miR-34c. Methylation of the miR-34b/c CpG island was frequently observed in GC cell lines (13/13, 100%) but not in normal gastric mucosa from healthy *H. pylori*-negative individuals. Transfection of the precursors of miR-34b and miR-34c into GC cells suppressed growth and changed the gene expression profile. Methylation of miR-34b/c was found in a majority of primary GCs (83/118, 70%). Notably, analysis of the non-cancerous gastric mucosae from GC patients (n = 109) and healthy individuals (n = 85) revealed that methylation levels were higher in the gastric mucosae of patients with multiple GC lesions than in the mucosae from those patients with single GC and the mucosae from healthy *H. pylori*-positive individuals. These results suggest that miR-34b and miR-34c are novel tumor suppressors frequently silenced by DNA methylation in GC. Methylation of miR-34b/c appears to be significantly involved in an epigenetic field defect in the stomach and to be a useful biomarker to identify individuals at high risk for multiple GC.

Methylation of miR-34b/c in the mucosa of the noncancerous gastric body may be a useful biomarker for predicting the risk of metachronous GC

Metachronous GC can develop after endoscopic resection of GC and is not predictable based on the clinical characteristics alone. Aberrant DNA methylation in noncancerous gastric mucosa has been implicated in gastric carcinogenesis and may be a useful biomarker of GC risk. Suzuki *et al*[55] evaluated the clinical utility of DNA methylation as a biomarker of metachronous GC risk. Scheduled follow-up endoscopy was performed in 129 patients after curative endoscopic resection of early GC. Biopsy specimens were collected from noncancerous mucosa in the gastric antrum and body. A quantitative methylation analysis of miR-34b/c, SFRP1, SFRP2, SFRP5, DKK2 and DKK3 using bisulfite pyrosequencing was performed on the collected biopsy specimens. The utility of the methylation status for predicting the risk of developing metachronous GC was analyzed using Kaplan-Meier and Cox proportional hazards models. During the follow-up period, 17 patients (13%) developed metachronous GCs. The cumulative incidence of metachronous GC was significantly higher among patients with elevated miR-34b/c, SFRP2 and DKK2 methylation in the gastric body. Elevated methylation of miR-34b/c showed the most significant association with the risk of metachronous GC; the cumulative incidence of metachronous GC was much higher in the high miR-34b/c-methylation group than in the low methylation group. Multivariate analysis adjusted for age, sex, *H. pylori* status and pathological findings showed that miR-34b/c methylation in the gastric body was an independent predictor of metachronous GC risk. Methylation of miR-34b/c in the mucosa of the noncancerous gastric

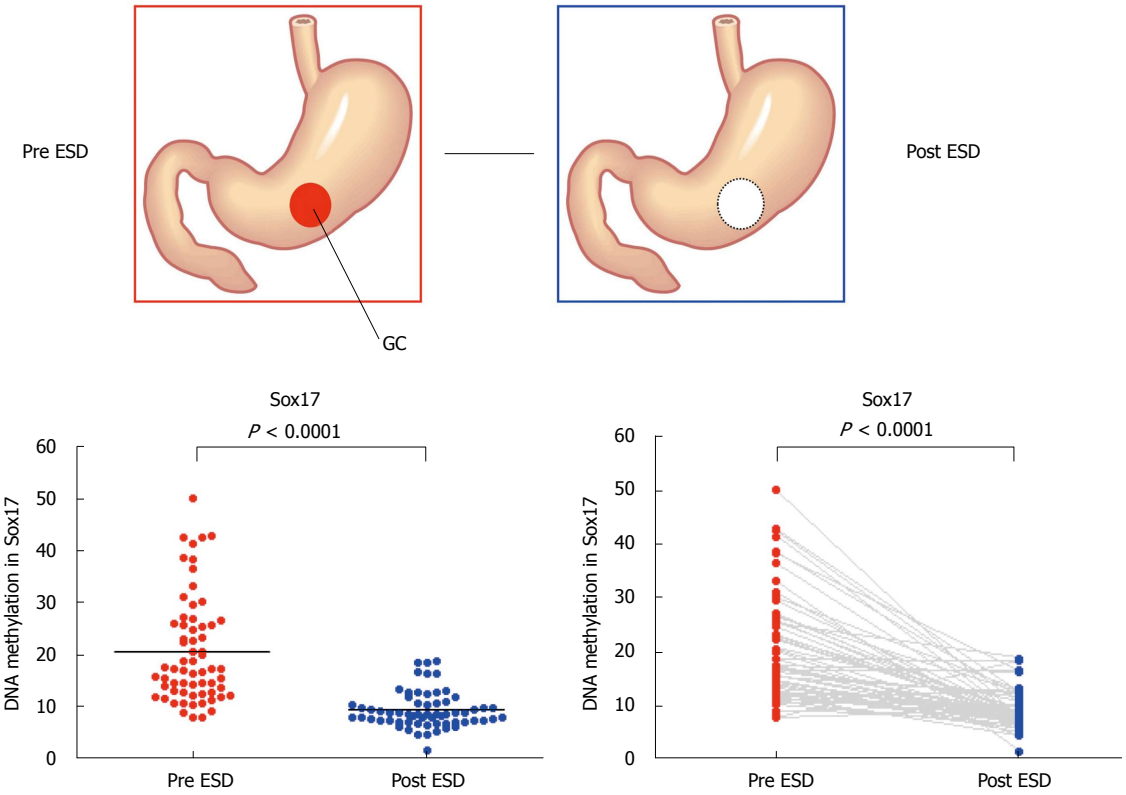

Figure 3 Methylation levels of Sox17 before and after endoscopic submucosal dissection. Methylation levels of Sox17 were analyzed by pyrosequencing using the DNA recovered from gastric washes before and after endoscopic submucosal dissection[57].

body may be a useful biomarker for predicting the risk of metachronous GC. Finally, NGS technologies may characterize an epigenetic field defect more clearly and highlight more useful biomarkers.

Sensitive and specific detection of early GC by DNA methylation analysis of gastric washes

Because many mucosal cells can be found in the gastric juice, the detection of molecular markers in the gastric juice was a possible noninvasive approach to detect GC. However, the use of gastric juice as a molecular diagnostic or predictive tool has been previously reported to be impractical because the DNA is easily degraded by gastric acidity. In this regard, Watanabe *et al*[56] have developed a new method for GC detection by DNA methylation in gastric washes but not in gastric juice. These authors analyzed 51 candidate genes in 7 GC cell lines and 24 GC samples (training set). They then selected 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5* and *MLF1*) for further analyses. The methylation status of these genes was analyzed in a test set consisting of 131 GCs at various stages. The 6 candidate genes were validated in a different population of 40 primary GC samples and 113 noncancerous gastric mucosa samples. The 6 genes showed differential methylation in GC and normal mucosa in the training, test and validation sets. GDNF and MINT25 were the most sensitive molecular markers of early-stage GC, whereas PRDM5 and MLF1 were markers of a field defect. A close correlation between methylation levels in tumor biopsy samples and gastric washes was noted. MINT25 methylation showed the best sensitivity (90%) and specificity (96%), and it had the greatest area under the receiver operating characteristic curve (0.961) in terms of tumor detection in gastric washes. MINT25 methylation in gastric washes may be a sensitive and specific marker for the screening of GC.

Detection of early GC by DNA methylation analysis of Sox17 in gastric washes

Although minimally invasive treatment is widely accepted for early-stage GC, appropriate risk markers to detect residual cancer after endoscopic resection and the potential for recurrence are not available. To find candidate genes that might be markers for the detection of early GC, Oishi *et al*[57] performed methylated CpG island amplification microarray analysis on 12 gastric washes (from the pre- and post-endoscopic treatment of six patients). Among the candidate genes, the *Sox17* gene was selected for further analysis. The DNA methylation status of Sox17 was examined in a validation set consisting of 128 gastric wash samples (64 pre-treatment and 64 post-treatment) from cases of early GC. Sox17 showed significant differential methylation in the pre- and post-treatment gastric washes of early GC patients (Figure 3). Moreover, the treatment of GC cells that lacked Sox17 expression with the methyltransferase inhibitor 5-aza-2'-deoxycytidine restored the gene's expression. Additionally, the introduction of exogenous Sox17 into silenced

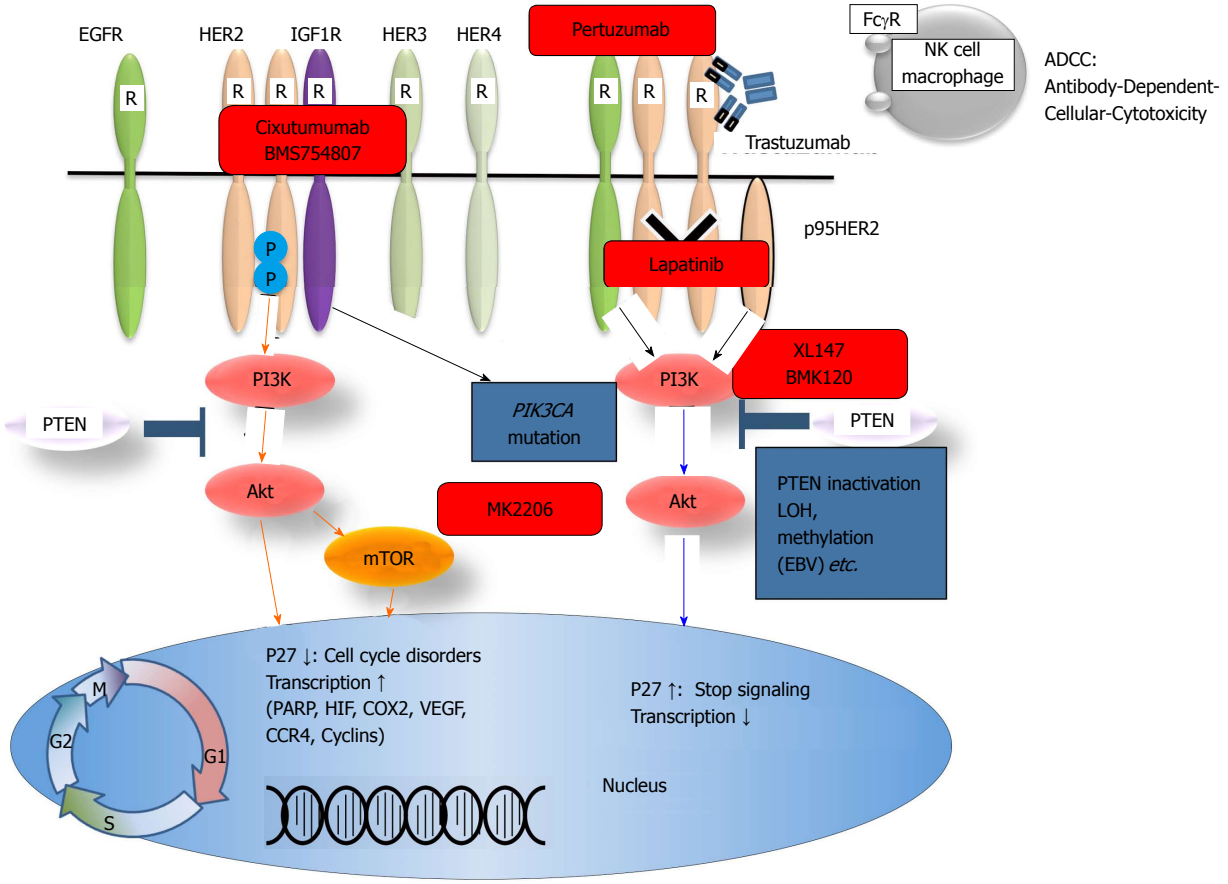

Figure 4 Human epidermal growth receptor family members, the PI3K/Akt pathway, and targeted drugs. HER: Human epidermal growth receptor; NK: Natural killer; IGF1R: α-insulin-like growth factor 1-receptor; EGFR: Epidermal growth factor receptor; PI3K: Phosphatidylinositol-3-kinase; PTEN: Phosphatase and tensin homologue.

GC cells suppressed colony formation. The data suggest that the silencing of Sox17 occurs frequently in early GC and plays a key role in the disease. Gastric wash-based DNA methylation analysis could be useful for the early detection of recurrence following endoscopic resection in early GC patients. Interestingly, the usefulness of gastric wash-based molecular testing for antibiotic resistance in *H. pylori* has also been reported[58]. It will be interesting to analyze gastric washes using NGS.

Anti-HER2 antibody trastuzumab has led to an era of personalized therapy in GC

Trastuzumab is an antibody that targets the HER2 extracellular domain and induces antibody-dependent cellular cytotoxicity and inhibition of the HER2 downstream signals (Figure 4). In the ToGA study, standard chemotherapy regimens (capecitabine plus cisplatin or fluorouracil plus cisplatin) combined with trastuzumab resulted in a longer survival time than standard regimens without trastuzumab in patients with HER2-positive GC[59]. Thus, HER2 expression has become a major concern in GC[60]. HER2 overexpression is observed in 7%-34% of GC cases. Mechanisms of resistance to trastuzumab have been reported in breast cancer. There are various mechanisms underlying trastuzumab resistance, such as alterations of the HER2 structure or surroundings, dysregulation of HER2 downstream signal effectors and interaction of HER2 with other membrane receptors (Figure 4). The PI3K-Akt pathway is one of the main downstream signaling pathways of HER2. It is well known that PIK3CA mutations and PTEN inactivation cause over-activation of a downstream signal without activation of an upstream signal. The frequencies of PIK3CA mutations and PTEN inactivation in GC have been reported to be 4%-25% and 16%-77%, respectively. However, little is known about the association between HER2 expression and PI3K-Akt pathway alterations in GC. Sukawa *et al*[29] have found that HER2 overexpression was significantly correlated with pAkt expression in GC tissues. Furthermore, pAkt expression was correlated with poor prognosis. These results suggest that the PI3K-Akt pathway plays an important role in HER2-positive GC. Moreover, PIK3CA mutations and PTEN inactivation could affect the effectiveness of HER2-targeting therapy. Thus, it is necessary to clarify not only HER2 alterations but also PI3K-Akt pathway alterations to optimize HER2-targeting therapy in patients with GC. In this regard, NGS will be useful for the identification of complicated mechanisms of trastuzumab resistance in GC. The only approved targeted therapy for patients with advanced GC is trastuzumab. It is hoped that NGS will reveal a driver gene alteration that will make other targeted

therapies possible[13,61].

Monoclonal antibodies targeting VEGF (AVAGAST trial) and VEGFR-2 (REGARD trial) in advanced GC

Several vascular endothelial growth factor (VEGF)-targeted agents have been developed, including neutralizing monoclonal antibodies (MoAbs) to VEGF/VEGFRs, soluble VEGF receptors and tyrosine kinase inhibitors (TKIs). The anti-VEGF MoAb bevacizumab has been approved for colorectal cancers. VEGF and VEGF receptor-2 (VEGFR-2)-mediated signaling and angiogenesis contribute to the pathogenesis and progression of GC. The Avastin in Gastric Cancer (AVAGAST) trial was a multinational, randomized, placebo-controlled trial designed to evaluate the efficacy of adding bevacizumab to capecitabine-cisplatin in the first-line treatment of advanced GC[62]. The study showed that adding bevacizumab to the chemotherapy regimen in patients with advanced GC improved the progression-free survival and tumor response rate but not the overall survival. A following biomarker evaluation analysis revealed that plasma VEGF-A and tumor neuropilin-1 are strong biomarker candidates for predicting the clinical outcome in patients with advanced GC treated with bevacizumab[63]. In this regard, NGS will be a powerful method for the identification of predictive biomarkers.

To analyze whether ramucirumab, a monoclonal antibody targeting VEGFR-2, prolongs survival in patients with advanced GC, an international, randomized, double-blind, placebo-controlled, phase 3 trial was conducted in 29 countries[64]. In total, 355 patients with advanced gastric or gastro-esophageal junction adenocarcinoma and disease progression after first-line chemotherapy were randomly assigned (2:1) to receive best supportive care plus either ramucirumab 8 mg/kg (n = 238) or placebo (n = 117), intravenously once every 2 wk. The primary endpoint was overall survival. The median overall survival was 5.2 mo in the ramucirumab group and 3.8 mo in the placebo group (HR = 0.776, 95%CI: 0.603-0.998, P = 0.047). The survival benefit with ramucirumab remained unchanged after multivariate adjustment for other prognostic factors (multivariate HR = 0.774, 95%CI: 0.605-0.991, P = 0.042). Thus, ramucirumab is the first biological treatment given as a single drug that showed survival benefits in patients with advanced gastric or gastro-esophageal junction adenocarcinoma who progressed after first-line chemotherapy. The findings also validate VEGFR-2 signaling as an important therapeutic target in advanced GC.

Potential targeted drugs for GC

Using NGS to target a subset of druggable genes becomes a more effective way to discover therapeutic targets[13,14,61]. There are several potential targeted drugs, either MoAb or small-molecule TKIs, that are being investigated either in synergy with, or in place of, established treatments. These drugs include inhibitors of growth factors and their receptors [*i.e.*, VEGF, epidermal growth factor receptor, HER2, insulin-like growth factor 1 (IGF1) receptor, c-MET], MEK inhibitors and drugs targeting the Hedgehog pathway[65].

Dysregulation of the IGF1 and IGF2/IGF1R system has been implicated in the pathogenesis of GC[66-69]. The expression levels of both IGFs and IGF1R are increased in GC. IGF1R is also involved in angiogenesis and lymphangiogenesis through the modulation of VEGF expression in a GC cell line[70]. IGF1R blockade reduced tumor angiogenesis and enhanced the effects of bevacizumab in a GC cell line. Thus, targeting IGF1R in combination with agents that block the VEGF pathway may have therapeutic utility in GC. Moreover, targeting the novel miR-7/IGF1R/Snail axis has been reported to be useful as a therapeutic approach to block GC metastasis[71].

CONCLUSION

The genetic and epigenetic alterations in GCs continue to inspire biological and clinical implications. Recent advances in the molecular study of GC have brought new diagnostic and therapeutic strategies into clinical settings. The advantages of using DNA methylation as a biomarker for the detection of GC in biopsy specimens and non-invasive body fluids such as serum and gastric washes may have a possible clinical application in GC. Further analysis is required to gain a deeper insight into GC carcinogenesis, a better understanding of disease pathogenesis and the development of new diagnostic and therapeutic approaches targeting essential pathogenic alterations. In this regard, the rapid advances in NGS technologies will hopefully continue to reveal driver alterations of GC, further our understanding of gastric carcinogenesis and improve the therapy for each individual tumor. The characterization of genes that were discovered by NGS rather than by laboratory and clinical research is also necessary.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Endoscopic submucosal dissection for undifferentiated-type early gastric cancer: Do we have enough data to support this?

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Abstract

Although endoscopic submucosal dissection (ESD) is now accepted for treatment of early gastric cancers (EGC) with negligible risk of lymph node (LN) metastasis, ESD for intramucosal undifferentiated type EGC without ulceration and with diameter ≤ 2 cm is regarded as an investigational treatment according to the Japanese gastric cancer treatment guidelines. This consideration was largely based on the analysis of surgically resected EGCs that contained undifferentiated type EGCs; however, results from several institutes showed some discrepancies in sample size and incidence of LN metastasis. Recently, some reports about the safety and efficacy of ESD for undifferentiated type EGC meeting the expanded criteria have been published. Nonetheless, only limited data are available regarding long-term outcomes of ESD for EGC with undifferentiated histology so far. At the same time, endoscopists cannot ignore the patients' desire to guarantee quality of life after the relatively non-invasive endoscopic treatment when compared to conventional surgery. To satisfy the needs of patients and provide solid evidence to support ESD for undifferentiated EGC, we need more delicate tools to predict undetected LN metastasis and more data that can reveal predictive factors for LN metastasis.

Key words: Early gastric cancer; Endoscopic submucosal dissection; Undifferentiated histology; Indications

Core tip: Endoscopic submucosal dissection (ESD) for intramucosal undifferentiated (UD) type early gastric cancer (EGC) without ulceration and with diameter ≤ 2 cm is regarded as an investigational treatment according to the Japanese gastric cancer treatment guidelines. In contrast, the controversial results about the safety of ESD for UD-EGC fulfilling the criteria have been reported and a little is known about the long-term outcomes. Therefore, in this review, we focused on the safety and therapeutic efficacy of ESD for UD-EGC with reference to risks for lymph node metastasis within the proposed criteria as well as the short-term and long-term outcomes of ESD for UD-EGC.

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INTRODUCTION

Early gastric cancer (EGC) is defined as gastric cancer that is confined to the mucosa or submucosa, irrespective

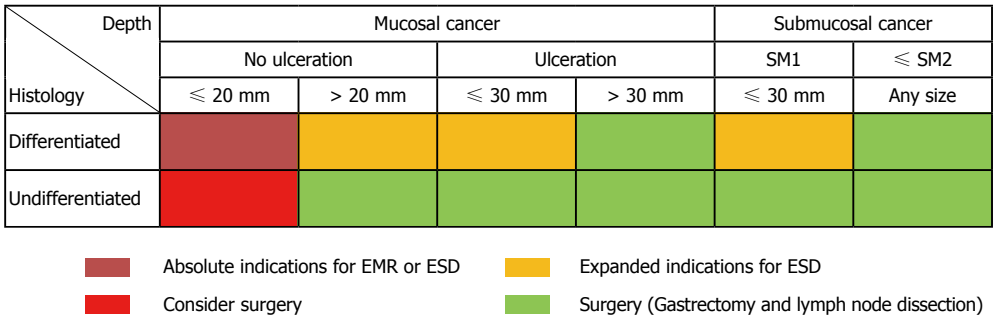

Figure 1 Absolute and expanded indication for endoscopic mucosal resection and endoscopic submucosal dissection for early gastric cancer. SM1: Tumor invasion into the upper third of the submucosa (≤ 500 μm); SM2: Tumor invasion into the mid-third of the submucosa (> 500 μm). EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

of the presence of regional lymph node (LN) metastases[1]. In the Eastern hemisphere, up to 70% of all gastric cancers are diagnosed as EGCs (due to mass population screening)[2-4], whereas in the Western hemisphere, the rate of gastric cancers identified as EGCs accounts for only about 15%[5,6]. EGC reveals a favorable prognosis compared with advanced gastric cancer, with 5-year survival rates being in excess of 90% to 95%, based on Korea, Japan, and European data[7-13].

In Eastern countries, endoscopic resection (ER), including endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD), has been widely accepted as a minimally invasive treatment for EGC with a negligible risk of LN metastasis[14-18]. Recently, considerable data have also been reported from the Western world as ER is gaining wide acceptance[19-21]. Tumors indicated for ER as a standard treatment are differentiated-type adenocarcinomas without ulceration, of which the depth of invasion is clinically diagnosed as mucosal layer and the diameter is ≤ 20 mm[22]. Gotoda *et al*[23] studied surgically resected specimens of EGC and suggested the following four expanded indication criteria for endoscopic treatment of EGC without LN metastasis: (1) differentiated intramucosal cancer without ulceration, regardless of size; (2) differentiated intramucosal cancer with ulceration and diameter ≤ 30 mm; (3) differentiated minute submucosal penetrative cancer in diameter ≤ 30 mm; and (4) undifferentiated (UD) type intramucosal cancer without ulceration and diameter ≤ 20 mm. In particular, surgery was still considered in the UD-EGC meeting the expanded criteria because endoscopic *en-bloc* removal was sometimes difficult in this type of tumors (Figure 1)[24,25]. However, Hirasawa *et al*[26] added to the body of evidence that there is no LN metastasis in patients with UD-EGC within the expanded criteria. This study revealed the 95%CI of the calculated risk of metastasis to nodes was 0%-0.96%, while the earlier study by Gotoda *et al*[23] showed that of risk was 0%-2.6% due to small sample size (n = 141), which may potentially be inferior to the outcomes of surgical resection.

Along these lines, the Japanese gastric cancer treatment guidelines (2010, ver. 3) state that ER for these UD-EGCs is regarded as an investigational treatment, and that ESD, not EMR, should be employed. In contrast, clinical practice guidelines, according to both National Comprehensive Cancer Network[27] and European Society for Medical Oncology[28], do not yet recognize ER for EGCs meeting the expanded criteria as safe. Moreover, the controversial results about the safety of ESD for UD-EGC fulfilling the criteria have been reported and a little is known about the long-term outcomes. Therefore, in this review, we focused on the safety and therapeutic efficacy of ESD for UD-EGC with reference to risks for LN metastasis within the proposed criteria as well as the short-term and long-term outcomes of it.

PREOPERATIVE ASSESSMENT OF LN METASTASIS

The most important factor concerning endoscopic treatment with curative intent is the prediction of regional LN metastasis before treatment[22,27,28]. Reported rates of LN metastasis in EGC range from 5.7% to 20% based on the analysis of surgically resected specimen of EGC[29-34]. UD-EGC demonstrates 4.2% to 4.9% and 19.0% to 23.8%of LN metastasis in the mucosal and submucosal invasive tumors, respectively[23,26]. To date, no imaging modality has been proven to be consistently accurate in assessing LN metastasis in EGC[35,36]. Endoscopic ultrasound (EUS) is one of most studied procedures for the locoregional staging of gastric cancer. Reported sensitivities and specificities of EUS to detect LN metastases in gastric cancers varied widely, between 16.7% and 95.3%, and between 48.4% and 100%, respectively[35]. EUS demonstrated a moderate accuracy that seems to describe advanced T stage (T3 and T4) better than N or less advanced T stage[37,38]. Although a clinically relevant benefit of EUS to distinguish intramucosal lesions from submucosal lesions should be further improved[39], EUS is an important imaging modality for preoperative assessment to exclude LN metastasis as well as to confirm deeper wall invasion including the proper muscle layer. Nevertheless, we should consider that UD histology would cause under-diagnosis and affect the accuracy of EUS compared to the differentiated histology[40].

In addition to the diagnostic role of magnifying endoscopy with narrow-band imaging (ME-NBI) for determining tumor margin in EGC[41,42], ME-NBI has been suggested as a supporting tool for the assessment of

Table 1 Curability for endoscopic resection of early gastric cancer

Curability criteria	
Curative resection	*En bloc* resection, no lateral and vertical margin positivity, no lymphovascular invasion Intramucosal cancer, differentiated histology, size ≤ 20 mm, No ulcerative finding
Curative resection for expanded indications	*En bloc* resection, no lateral and vertical margin positivity, no lymphovascular invasion Intramucosal cancer, differentiated histology, size > 20 mm, no ulcerative finding Intramucosal cancer, differentiated histology, size ≤ 30 mm, presence of ulcerative finding SM 1 depth of invasion, differentiated histology, size ≤ 30 mm, no ulcerative finding Intramucosal cancer, undifferentiated histology, size ≤ 20 mm, no ulcerative finding
Non-curative resection	Any resection that does not satisfy one of the above criteria

SM: Submucosa.

invasion depth in EGC[43-46]. In contrast to the usefulness of ME-NBI for evaluating invasive depth in esophageal or colon cancer[47,48], the utility of ME-NBI for determining invasion depth in EGC is not conclusive, because the invasive tumor is often not exposed at the surface and the mucosal structure remains, even when cancer invades the submucosa. Therefore, it is difficult to estimate reliably the depth of invasion by surface appearance[49]. ME-NBI should also distinguish findings suggestive of submucosal invasion from those indicative of the UD histologic type[44,45]. The findings of a nonstructural pattern in the neoplastic lesion of the stomach on ME[45] or no surface pattern and sparse microvessels (markedly distorted, isolated, heterogeneous) or with avascular areas on ME-NBI[44] are indicative of undifferentiated type adenocarcinoma or differentiated cancer with deep submucosal invasion. In contrast, ME-NBI images of UD-EGC were very closely related to the histopathological findings in other study[50], and therefore, this imaging tool can be useful in the pretreatment assessment of the histopathological patterns of cancer development and the lateral extent of UD-EGC. Thus, the role of ME-NBI in differentiation of histologic types in addition to invasive depth should be validated through further prospective studies.

Other imaging modalities including abdominal ultrasound (AUS), computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) achieved limited success to stage preoperative LN status[35,36]. A meta-analysis by Seevaratnam *et al*[36] showed that imaging modalities range in overall accuracy from 53.4% (MRI) to 68.1% (AUS), in sensitivity from 40.3% (PET) to 85.3% (MRI), and in specificity from 75.0% (MRI) to 97.7% (PET), with no significant differences between modalities. To date, there are no clinically relevant imaging tools to detect the submucosal invasion and the LN metastasis in EGC that are critical conditions for determining proper candidates for ER.

RISK FACTORS FOR LN METASTASIS AND PROPOSED CRITERIA FOR ESD

Because currently available imaging modalities fail to accurately evaluate nodal status, endoscopic resectability according to nodal status in EGC and subsequent curability are still determined by means of the presence or absence of certain tumor characteristics which were obtained from the analysis of surgically resected EGC. According to the Japanese gastric cancer treatment guidelines[22], the main risk factors predictive of LN metastasis in EGC are histologic type, depth of invasion, ulceration, size, and lymphovascular invasion largely based on two large-scale datasets[23,26]. These factors consist of absolute and expanded indications as well as curability of ER with *en bloc* resection and negative lateral/vertical margin (Table 1)[22,51]. A meta-analysis by Kwee *et al*[52] identified the characteristics related to LN metastasis in EGC, including age, gender, location, size, macroscopic type, ulceration, histologic type in accordance with Japanese and Lauren classification, lymphovascular invasion, submucosal vascularity, a proliferating cell nuclear antigen labeling index, a matrix metalloproteinase-9-positivity, a gastric mucin phenotype, and a vascular endothelial growth factor-C-positivity. These factors revealed partially different correlations with LN metastasis in intramucosal and submucosal EGCs, respectively.

With regard to LN metastasis particularly in UD-EGC, many recent studies investigated the risk factors and suggested their criteria for ER of UD-EGC (Table 2)[23,26,53-65]. The overall rates of LN metastasis in UD-EGC varied from 7.9% to 24.5%; however, the heterogeneous composition in subtypes of UD histology in lesions from 15 studies should be taken into account.

Size of lesion

Although the intramucosal lesions without ulceration and diameter ≤ 20 mm have been considered as rational criteria for ESD in UD-EGC by Japanese researchers[23,26], different ER criteria have also been suggested with various standards in size, depth of invasion, and presence of ulcerative finding[53-65]. Concerning lesion size, a majority of recent studies (11/15, 73.3%) suggested that a diameter of 20 mm to 30 mm would be the upper limit of the size criterion for UD-EGC to be amenable to treatment with ESD; however, the remaining four studies proposed a diameter of 10 or 15 mm as the upper limit of the criterion, based on their results suggesting the possibility of LN metastasis even in smaller UD-EGC[54,60,64,65]. Debates over the size criterion were highlighted by several reports of LN metastasis of UD-EGCs within the expanded criteria, including a diameter ≤ 20 mm[31,66-70]. Moreover, the size discrepancy between pathologic size and endoscopic size should be resolved, because we can only determine the indications of ER based on the endoscopically estimated size. While a previous study revealed that endoscopic visual estimation method was found to show

Table 2 Proposed criteria for endoscopic resection of undifferentiated type early gastric cancer

Study	Year	Country	No. of patients with UD-EGC	No. of patients with PD/SRC/MC	LNM in UD-EGC, *n* (%)	LNM in proposed criteria	Risk factors related to LNM in UD-EGC	Proposed criteria of ER for UD-EGC			
								Size, mm	Depth of invasion	Ulcer	LVI
Tong *et al*[53]	2011	China	193	81/102/7/3[1]	46 (23.8)	0/72	Size, depth of invasion, LVI, Histologic type	NS or ≤ 20	M or SM	NS	No
Kim *et al*[54]	2011	South Korea	707	288/419/0	65 (9.2)	0/101	Size, depth of invasion, LVI, Age[2]	15[3]	M	NS	No
Li *et al*[55]	2010	China	108	85/16/7	16 (14.8)	0/25	Size, depth of invasion, LVI	20	M	NS	No
Park *et al*[56]	2009	South Korea	215	Only SRC	17 (7.9)	0/57	Depth of invasion, LVI	25	SM2	NS	No
Kunisaki *et al*[57]	2009	Japan	573	182/378/13	74 (12.9)	0/85	Size, depth of invasion, LVI	20	M	NS	No
Hirasawa *et al*[26]	2009	Japan	3843	NA	504 (13.1)	0/310	Size, depth of invasion, LVI	20	M	No	No
Hanaoka *et al*[58]	2009	Japan	143	NA	35 (24.5)	0/41	Size, depth of invasion, LVI, Histologic type	30	≤ 500 μm[4]	NS	No[5]
Ye *et al*[59]	2008	South Korea	591	266/316/9	79 (13.4)	0/119	Size, depth of invasion, LVI	25	M	NS	No
Park *et al*[60]	2008	South Korea	234	Only PD	25 (21.6)	0/56	Size, depth of invasion, LVI	15	M or ≤ 500 μm	NS	No
Li *et al*[61]	2008	China	85	Only PD	12 (14.1)	0/25	Size, depth of invasion, LVI	20	M	NS	No
Li *et al*[62]	2008	South Korea	646	307/330/9	61 (9.4)	1/201	Size, depth of invasion, LVI	20	M	NS	No
Ha *et al*[63]	2008	South Korea	641	248/388/5	100 (15.6)	0/77	Size, depth of invasion, LVI, histologic type	20	M	NS	No
Hyung *et al*[64]	2004	South Korea	289	NA	43 (14.9)	NA	Size, depth of invasion, LVI, histologic type	15	M	NS	No
Abe *et al*[65]	2004	Japan	175	68/104/3	32 (18.3)	0/6	Size, LVI	10	M	NS	No
Gotoda *et al*[23]	2000	Japan	2341	NA	243 (10.4)	0/141	Size, depth of invasion, LVI, histologic type, ulcer, macroscopic type	20	M	No	No

[1]Three patients had EGCs with histology of undifferentiated adenocarcinoma; [2]Young age less than 45 years was related to the lymph node metastasis of only poorly-differentiated carcinoma; [3]Size criteria were ≤ 25 mm in poorly-differentiated adenocarcinomas and ≤ 15 mm in signet-ring cell carcinomas, respectively; [4]The depth of invasion in proposed criteria was ≤ 500 μm or no more from the lower margin of the muscularis mucosae; [5]Hanaoka et al also suggested the proportion of undifferentiated components < 50% as one of criteria. UD-EGC: Undifferentiated type early gastric cancer; PD: Poorly-differentiated adenocarcinoma; SRC: Signet-ring cell carcinoma; MC: Mucinous carcinoma; LNM: Lymph node metastasis; ER: Endoscopic resection; LVI: Lymphovascular invasion; M: Mucosa; SM: Submucosa; NS: Not significant; NA: Not available.

reliable agreement with pathologic measurements in EGC treated with ER[71], other earlier ESD series showed the mean size discrepancies ranged from 5.8 mm to 6.8 mm, which are not negligible in ER for EGC[72,73]. In UD-EGC, the margins of the lesion tend to be obscured compared to the differentiated histology, which was found to cause frequent margin failure of ESD in our previous report[74]. Thus, a standard reliable measurement method is required through further prospective studies[75].

Submucosal invasion

Some studies suggest that a shallow submucosal invasion is an acceptable depth of invasion in ESD for UD-EGC[53,56,58,60]. However, this suggestion should be reserved until EUS is more reliable for determination of invasive depth, because there is a high chance of endosonographically underestimated depth of invasion and subsequently higher vertical margin positivity in poorly-differentiated EGC[40,74], in addition to the difficult assessment of depth of invasion in UD-EGC[76-79]. Additionally, the numbers of enrolled UD-EGCs in these studies, suggesting a minute submucosal invasion as a criterion for ER, were relatively small compared with other studies. More importantly, the majority of recent studies reported the LN metastasis in a depth of submucosal invasion[23,26,54,55,57,59,61-65].

Ulceration

Ulceration within the lesion is the representative finding with heterogeneity. More than moderate heterogeneity was identified at previous meta-analysis with possible explanation for this heterogeneity due to the interobserver variability between studies for the assessment of tumor ulcerations[52]. Furthermore, this may be due to the different definitions in addition to the interobserver variability for the assessment of ulcerations[52,67,75,80]. Though most of the recent studies (13/15, 86.7%) did not consider the ulcer finding in their proposed criteria, patients with tumor ulcerations had a significantly higher risk of LN metastasis in intramucosal EGC irrespective of histological type at meta-analysis[52]. And ulcerous change decreases the accuracy of EUS diagnosis for the invasive depth of EGC[81]. Therefore, we do not consider ER for UD-EGCs with ulceration as safe.

Table 3 Clinical characteristics of representative studies on endoscopic resection for undifferentiated type early gastric cancer

Study	Year	Country	No. of patients with UD-EGC	No. of patients with PD/SRC	Age (yr)[1]	Sex (male)	SM invasion	Ulcer	Size (mm)[1]	Size > 20 mm
Kim *et al*[80]	2013	South Korea	74	55/19	61.8 ± 12.0	40 (54.1)	16 (21.6)	11 (14.9)	19.9 ± 12.5	36 (48.6)
Abe *et al*[87]	2013	Japan	97	18/77/2[2]	62.0 (35.0-88.0)[3]	55 (56.7)	19 (19.6)	9 (9.3)	12.0[3]	14 (14.4)
Park *et al*[88]	2012	South Korea	77	47/15[4]	60.9 (33.0-82.0)	49 (63.6)	12 (15.6)	4 (5.2)	23.3 ± 14.0	35 (45.5)
Okada et al[89]	2012	Japan	103[5]	12/91	59.0 (34.0-91.0)	48 (46.6)	10 (9.7)	1 (1.0)	8.0 (1.0-33.0)[3]	NA
Kamada *et al*[76]	2012	Japan	46	NA	65.5 (29.0-90.0)	24 (52.2)	7 (15.2)	1 (2.2)	NA	8 (17.4)
Yamamoto *et al*[90]	2010	Japan	58	48/10	64.0 (33.0-81.0)	31 (53.4)	7 (12.1)	2 (3.4)	11.0 (2.0-28.0)	5 (8.6)
Kang *et al*[73]	2010	South Korea	60	30/30	56.7 ± 10.4	31 (51.7)	17 (28.3)	17 (28.3)	26.3 ± 12.9	31 (51.7)
Kim *et al*[74]	2009	South Korea	58	17/41	55.0 (26.0-81.0)	26 (44.8)	NA	0 (0)	13.3 ± 6.5	4 (6.9)

Data are expressed as absolute numbers (percentage) or mean ± SD. [1]Data are expressed as mean with standard deviation or range; [2]Two patients had EGCs with histology of moderately to poorly differentiated adenocarcinoma; [3]Data are expressed as median with or without range; [4]Fifteen patients had EGCs with mixed type histology; [5]A total of 103 EGCs in 101 patients were enrolled. UD-EGC: Undifferentiated typr early gastric cancer; PD: Poorly-differentiated adenocarcinoma; SRC: Signet-ring cell carcinoma; SM: Submucosa; NA: Not available.

Lymphovascular invasion

Only the absence of lymphovascular invasion was the criterion included by all studies, which was consistent with the results of a meta-analysis revealing that lymphatic tumor invasion is the strongest predictor for LN metastasis in both mucosal and submucosal gastric cancer[52]. For this reason, EGCs with lymphovascular invasion in endoscopically resected specimen should be treated by further surgery[22]. However, the Japanese gastric cancer treatment guidelines are not based on the status of lymphovascular invasion. The lymphovascular invasion is involved in the decision of curability of ER, since its evaluation can only be available in specimens obtained by ER. Moreover, the determination of lymphovascular invasion sometimes lacks objectivity possibly because of the inability to distinguish lymphatics from blood vessels on conventional hematoxylin-eosin staining[82]. Several studies suggested an endoscopic elevated macroscopic type[83] and a stromal cell-derived factor-1α as risk factors of lymphovascular invasion[84] with reports of usefulness of immunohistochemical staining for detection[82,85,86]. Considering the importance of lymphovascular invasion for prediction of LN metastasis, prospective studies of preoperative prediction for lymphovascular invasion are warranted.

CLINICAL CHARACTERISTICS

Clinical characteristics of recent representative studies on ER for UD-EGC are summarized in Table 3[73,74,76,80,87-90]. All eight studies were analyzed retrospectively. The numbers of lesions ranged from 46 to 103 lesions and were not large enough to elicit conclusive results. Six studies performed solely ESD[73,76,80,87,89,90] and the rest carried out both EMR and ESD[74,88]. Inclusion criteria of these studies were based on the expanded criteria except those of two studies by Kim *et al*[80] and Kang *et al*[73]. The study by Kim *et al*[80] included patients who refused surgery and were treated by ESD as an experimental treatment. The study by Kang *et al*[73] included patients with UD-EGC with ulceration. Submucosal invasion and ulcers were noted in 9.7%-19.6% and 1.0%-9.3% of lesions satisfying the expanded criteria, respectively. The two studies that included patients who refused surgery and lesions with ulcerations in endoscopic finding showed relatively high submucosal invasion and ulceration rates. The inaccurate endoscopic size estimation in UD-EGCs is well noted in the studies, because the lesions with size > 20 mm were noted in up to 45.5% of lesions[88]. Particularly, the study including intramucosal UD-EGC with size ≤ 20 mm regardless of ulcerations revealed notably higher SM invasion (28.3%), ulcer finding (28.3%), and size > 20 mm (51.7%) rates[73]. The overall inaccuracies of assessment of depth of invasion, ulcerative findings, and size of UD-EGC tumors fulfilling the expanded criteria are not negligible, and thus ESD criteria based on endoscopic and histologic findings in UD-EGC should have more restrictions compared to differentiated EGC. To overcome this limitation, new methods beyond the current level of technology are strongly needed.

SHORT-TERM OUTCOMES

In addition to a very low possibility of LN metastasis, the safety of ESD for UD-EGC can be established based on the feasibility of curative resection with acceptable complication rates and consequently favorable long-term outcomes.

Short-term outcomes, including *en bloc* resection, complete resection, curative resection, and complication rates, of ER for UD-EGC are listed in Table 4[73,74,76,80,87-90]. Whereas homogeneous definitions of *en bloc* resection applied for the studies, the definitions of complete resection category were heterogeneous depending on the involvement of *en bloc* resection or lymphovascular invasion or submucosal invasion[73,74,80,87,90]. Additionally, the definitions of curative resection in some studies did not clarify the involvement of *en bloc* resection[80,89,90]. The overall rates of *en bloc* resection, complete resection, and curative resection of ER for UD-EGCs varied from 83.1% to 100%, from 55.0% to 90.7%, and from 31.1% to 82.5%, respectively, while those of ESD for UD-EGCs meeting the expanded criteria ranged from 91.3% to 99.0%, from 89.7% to 90.7%, and from 63.9% to 82.5%, respec-

Table 4 Short-term outcomes of endoscopic resection for undifferentiated early gastric cancer *n* (%)

Study	LMP	VMP	LVI	*En bloc* resection	Complete resection	Curative resection	OP after ER[1]	Residual tumor[2]	LNM[2]	Bleeding	Perforation
Kim *et al*[80]	NA	NA	10 (12.5)	67 (90.5)	54 (73.0)	23 (31.1)	19/51 (37.3)	NA	NA	1 (1.4)	3 (4.1)
Abe *et al*[87]	5 (5.2)	4 (4.1)	3 (3.1)	96 (99.0)	88 (90.7)	62 (63.9)	21/35 (60.0)	1/21 (4.8)	2/21 (9.5)	4 (4.1)	4 (4.1)
Park *et al*[88]	12 (15.6)[3]		5 (6.5)	64 (83.1)	NA	35 (45.5)	11/42 (26.2)	NA	0/11 (0.0)	NA	NA
Okada *et al*[89]	5 (4.9)[3]		2 (2.0)	102 (99.0)	NA	85 (82.5)	10/18 (55.6)	2/10 (20.0)	0/10 (0.0)	9 (8.7)	1 (1.0)
Kamada *et al*[76]	5 (10.9)	4 (8.7)	4 (8.7)	42 (91.3)	NA	NA	5	1/5 (20.0)	NA	2/46 (4.3)	2 (4.3)
Yamamoto *et al*[90]	1 (1.7)	0 (0.0)	2 (3.4)	57 (98.3)	52 (89.7)	46 (79.3)	8/12 (66.7)	2/8 (25.0)	0/8 (0.0)	5 (8.6)	2 (3.4)
Kang *et al*[73]	14 (23.3)	11 (18.3)	15 (25.0)	60 (100)	33 (55.0)	NA	15/27 (55.6)	6/15 (40.0)	2/15 (13.3)	1 (1.7)	1 (1.7)
Kim *et al*[74]	10 (52.6)	9 (47.4)	NA	49 (84.5)	39 (67.2)	NA	9/19 (47.4)	4/9 (44.4)	1/9 (11.1)	8 (13.8)	1 (1.7)

[1]Proportions are ratio of additional operation to incomplete or non-curative endoscopic resection; [2]Data are the incidence of residual tumor or lymph node metastasis in specimens obtained by additional operation; [3]Data are cases with lateral and/or vertical margin positivity. LMP: Lateral margin positivity; VMP: Vertical margin positivity; LVI: Lymphovascular invasion; OP: Operation; ER: Endoscopic resection; LNM: Lymph node metastasis; NA: Not available.

tively[76,87,89,90]. The results of ESD for cases within the expanded criteria were comparable with the outcomes of ESD for differentiated EGCs fulfilling the criteria of 93.0% to 95.7% and 81.0% to 91.1% for *en bloc* and complete resection rates, respectively[91-93]. In contrast, the curative resection rate seems to be lower than that of differentiated EGCs, which is 91.1%[93]. This may arise from less accurate endoscopic size estimation in UD-EGC due to an ill-defined margin of tumor infiltration[41,94,95] and several distinct features of UD-EGC, including a larger size and submucosal infiltration that can lead to higher rates of lymphovascular invasion[73,82,90,96-98], compared with EGCs with differentiated histology. Therefore, the achievement of reasonable curative resection rate in ESD for UD-EGC is critical by means of more precisely defining of curable lesions.

Further surgical treatments were performed in 26.2% to 60.0% of patients with incomplete or non-curative ER. The presence of residual tumor and LN metastasis in surgical specimens after incomplete or non-curative ER were detected in 4.8% to 44.4% and 0% to 13.3% of cases. The overall rates of bleeding and perforation varied from 1.4% to 13.8% and from 1.0% to 4.3%, respectively, whereas those of ESD for UD-EGCs meeting the expanded criteria ranged from 4.1% to 8.7% and from 1.0% to 4.3%, respectively. The results from lesions within the criteria were comparable with the bleeding and perforation rates of ESD for differentiated EGCs fulfilling the criteria, which were 2.1% to 4.9% and 2.4% to 6.6%, respectively[91-93]. In terms of procedure-related complications, ESD for UD-EGC appears not to be inferior to ESD for EGC with differentiated histology.

LONG-TERM OUTCOMES

Only limited data are available regarding long-term outcomes of ESD for UD-EGC[51,80,87,89], although the recurrences after ER have been shown in 0% to 6.9% with follow-up durations ranging from 16 to 45.6 mo[73,74,76,88,90]. Okada *et al*[89] reported the first study regarding long-term outcomes of ESD for UD-EGC with limited median follow-up periods. The 5-year cause-specific survival rate among 78 patients with curative resection of UD-EGC was 100%, which was as high as the reported data for gastrectomy[99,100]; however, the median follow-up period was only 36 mo. The cumulative 3- and 5-year disease-free survival rates are 96.7% (95%CI: 92.0%-100%) and 96.7% (95%CI: 92.0%-100%), respectively. During the follow-up period, all patients survived, and no cases of local recurrence and/or distant metastasis were observed. There were only second ESDs for one synchronous lesion of one patient 6 mo after the primary ESD (1/78, 1.3%) and two metachronous lesions of another patient after 23 mo (1/78, 1.3%).

Abe *et al*[87] analyzed the overall 5-year survival of 79 UD-EGC patients that underwent ESD, while they enrolled 97 patients for short-term outcomes analyses. Of the 46/79 patients in the long-term outcome group who had curative resection, none had local recurrence or LN or distant metastasis, and none died of gastric cancer during a median follow-up of 76.4 mo. The 5-year overall survival rate after curative resection was 93.0%, and no patient died of gastric cancer. These favorable results are comparable to long-term outcomes of those who underwent ESD for differentiated EGC and surgery for intramucosal gastric cancer, which have the overall survival rates of 92.4% to 97.1%[101-103] and 93.5%[104], respectively. The 5-year cumulative incidence of metachronous gastric cancer was 11.4% in the patients with curative resection and they were treated with ESD.

Kim *et al*[80] reported consistent results showing a local recurrence rate of 5.5% and a 5-year overall survival rate of 93.7% among 74 enrolled patients with median follow-up period of 34 mo (range 7-81 mo). All 4 recurred lesions did not meet the expanded indications and all underwent noncurative resection. There was no mortality related to ESD for treatment of EGC during follow-up, whereas a total of five patients died after ESD due to underlying diseases (four patients) and lung metastasis (one patient).

The questionnaire study on long-term outcomes of curative ESD for EGC at six Japanese institutions with follow-up rates of at least 90% over a minimum 5-year period was reported by Oda *et al*[51]. Of a total of 1289 patients with curative resections for the expanded indications, the long-term outcomes of 58 patients with

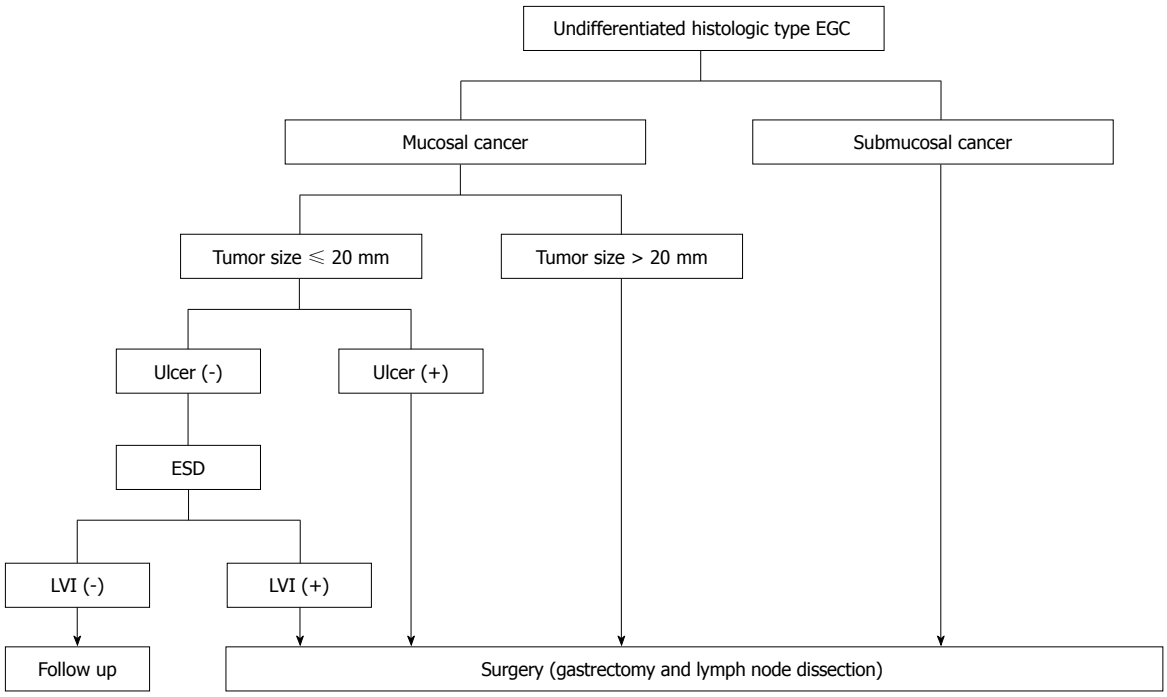

Figure 2 Treatment algorithm for undifferentiated type early gastric cancer according to depth of invasion, tumor size, ulceration, and lymphovascular invasion. EGC: Early gastric cancer; ESD: Endoscopic submucosal dissection; LVI: Lymphovascular invasion.

intramucosal UD-EGC ⩽ 20 mm in size without ulcerations were analyzed, and 96.6% of them (56/58) were followed up for at least 5 years. The overall mortality rate was 10.7% (6/56), and there was no local recurrence, or distant metastasis, or gastric cancer-related death during their long-term follow-up periods.

In addition to the 5-year survival outcomes, the long-term data on metachronous EGCs after ESD for UD-EGC are also lacking. The cumulative incidences of metachronous lesions varied from 1.3% to 11.4% during median follow-up periods with a range of 36-76.4 mo[87-89]. This finding is comparable to the annual incidences of metachronous lesions after ESD for differentiated EGC, which ranged from 1.9% to 3.9%[105,106] as well as reports of remnant gastric cancers occurring in 1.8% to 5% of patients who have had surgical treatment for gastric cancer[107,108]. Therefore, careful periodic endoscopic surveillance should be performed, because UD histology is a possible risk factor associated with the occurrence of metachronous lesions after ER[109]. Although the clinical importance of scheduled endoscopic surveillance after curative resection are recently evaluated through large-volume multicenter study[110], further studies on surveillance follow-up after curative ESD for UD-EGC, compared with curative cases in differentiated EGC, are warranted.

PROSPECTS FOR THE FUTURE

A combination of laparoscopic sentinel node biopsy and ESD for UE-EGC is an attractive option as a novel, whole stomach-preserved, minimally invasive approach with histological confirmation of LN metastasis. However, a number of technical controversies should be resolved to accept the laparoscopic sentinel node mapping and consequent intraoperative ESD as an acceptable treatment. These include the accuracy of intraoperative pathological diagnosis, the necessity of full-thickness resection, and the possibility of cancer cells being present in afferent lymphatic vessels leading to sentinel nodes[111]. In particular, a well-designed, multicenter feasibility study of laparoscopic sentinel node mapping and biopsy for UD-EGC should be conducted, though the accuracy of determining LN status by laparoscopic sentinel node biopsy is generally acceptable in cases with EGC[112-114].

Natural orifice transluminal endoscopic surgery (NOTES) is another promising area to supplement ESD by providing for the means for performing secure gastric closure at the time of the accidental perforation without recourse to surgical operation, or as a complement for endoscopic sentinel node biopsy[115-117]. The potential indications of NOTES have been suggested with a wide spectrum of upper gastrointestinal diseases, including submucosal malignancy and morbid obesity in female patients[118-120]. Furthermore, the first prospective study of 14 patients with EGC who had a risk for LN metastasis and who were treated by hybrid NOTES was reported and suggested that hybrid NOTES may be useful as a bridge between ER and laparoscopic surgery[121]. Nevertheless, given the relatively technical complexity and limits, NOTES has not been proven to remarkably superior to laparoscopic means so far.

CONCLUSION

Based on the results of studies on short- and long-term

outcomes, the expanded criteria for ESD of UD-EGC are feasible with reference to therapeutic efficacy and safety in the long-term period if curative resection is accomplished, although more long-term outcomes are needed. We now suggest the treatment algorithm for UD-EGC according to depth of invasion, tumor size, ulceration, and lymphovascular invasion (Figure 2). This is consistent with the conditions of curative resection according to the Japanese gastric cancer treatment guidelines[23,26]. However, we should recognize the limitation of current diagnostic and histological tools to predict LN metastasis. The innovative improvement of preoperative imaging modalities and well-defined criteria predictive of LN metastasis from multicenter, prospective studies would reduce the limitation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Somatic alterations in mitochondrial DNA and mitochondrial dysfunction in gastric cancer progression

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Abstract

Energy metabolism reprogramming was recently identified as one of the cancer hallmarks. One of the underlying mechanisms of energy metabolism reprogramming is mitochondrial dysfunction caused by mutations in nuclear genes or mitochondrial DNA (mtDNA). In the past decades, several types of somatic mtDNA alterations have been identified in gastric cancer. However, the role of these mtDNA alterations in gastric cancer progression remains unclear. In this review, we summarize recently identified somatic mtDNA alterations in gastric cancers as well as the relationship between these alterations and the clinicopathological features of gastric cancer. The causative factors and potential roles of the somatic mtDNA alterations in cancer progression are also discussed. We suggest that point mutations and mtDNA copy number decreases are the two most common mtDNA alterations that result in mitochondrial dysfunction in gastric cancers. The two primary mutation types (transition mutations and mononucleotide or dinucleotide repeat instability) imply potential causative factors. Mitochondrial dysfunction-generated reactive oxygen species may be involved in the malignant changes of gastric cancer. The search for strategies to prevent mtDNA alterations and inhibit the mitochondrial retrograde signaling will benefit the development of novel treatments for gastric cancer and other malignancies.

Key words: Gastric cancer; Somatic mitochondrial DNA mutations; Mitochondrial dysfunction

Core tip: In this review, we summarize recent somatic mitochondrial DNA (mtDNA) alterations identified in gastric cancer, and the relationship between these alterations and the clinicopathological features of gastric cancer. We suggest that point mutations and mtDNA copy number decreases are the two most common mtDNA alterations that potentially result in mitochondrial dysfunction in gastric cancer. Mitochondrial dysfunction-generated reactive oxygen species may be involved in the malignant changes of gastric cancer. The search for strategies to prevent the mtDNA alterations and inhibit the mitochondrial retrograde signaling will benefit the development of novel treatments for gastric cancer and other malignancies.

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INTRODUCTION

Gastric cancer is one of the most common causes of death in cancer patients throughout the world. Surgical resection with radical lymph nodes dissection is the primary therapy for gastric cancer[1]. Chemotherapy is an alternative treatment for unresectable gastric cancer or tumor recurrence after surgical resection. However, the response to chemotherapy remains unsatisfactory. Thus, it is important to identify novel drug targets and develop effective treatments for gastric cancer.

Based on the conceptual progress of the past decades, energy metabolism reprogramming was recently included as one of the cancer hallmarks[2]. Warburg[3,4] first proposed that tumor cells, unlike normal cells, exhibit increased glycolytic activity and reduced mitochondrial respiration even in the presence of oxygen. This phenomenon is known as the "Warburg effect". Increasing lines of evidence suggest that various molecular mechanisms generate the Warburg effect[5,6]. One of these mechanisms is mitochondrial dysfunction resulting from mutations in nuclear genes or mitochondrial DNA (mtDNA)[5-9].

Mitochondria are intracellular organelles in eukaryotic cells that participate in bioenergetics metabolism and cellular homeostasis, including the generation of ATP through respiration and oxidative phosphorylation (OXPHOS), the production of reactive oxygen species (ROS), and the initiation and execution of apoptosis[10]. Mitochondria contain multiple copies of mitochondrial DNA (mtDNA). Human mtDNA is a 16.6-kB double-stranded, circular DNA molecule that encodes 13 respiratory enzyme complex polypeptides, 22 transfer RNAs and 2 ribosomal RNAs required for mitochondrial protein synthesis[10]. Because mtDNA is essential for the maintenance of functionally competent organelles, the accumulation of mtDNA mutations or decreased mtDNA copy number is expected to affect energy production as well as enhance ROS generation and cell survival, and these processes may be involved in aging, mitochondrial diseases or cancer[9-12].

In the past decade, somatic mtDNA alterations have been identified in several types of cancer[8,9], including gastric cancer[13-15]. However, the role of these mtDNA alterations in tumorigenesis and cancer progression remains unclear. In this article, we review recent findings on somatic mtDNA alterations in gastric cancer. In addition, we discuss the potential factors that may lead to mtDNA mutations and propose a role of mtDNA alterations and mitochondrial dysfunction in the progression of gastric cancer.

SOMATIC MITOCHONDRIAL DNA ALTERATIONS IN GASTRIC CANCER

Several studies have identified various types of mtDNA alterations in gastric cancer[13-15], including point mutations, large-scale deletions, insertions, and copy number changes.

In one of our studies[15], 65% of the examined gastric cancer patients carried at least one mtDNA somatic point mutation. Among the identified point mutations, 69% occur in the D-loop region of mtDNA, 27% are found in the protein-coding region, and 4% are located in *tRNA* genes. Compared with other cancers, these mutations are similar in their incidence and distribution (Table 1)[15-38]. The D-loop region of mtDNA is the most frequent site of somatic mutation in cancers. Because the D-loop region contains the major regulatory sites for mtDNA replication and transcription, mutations near these sites might affect mtDNA copy number in cancers.

Given that the mtDNA D-loop region is a hot spot for somatic mutations in gastric cancer as well as other cancers, numerous studies focus on somatic mutations in this region[13,39-43]. The incidence of somatic mtDNA point mutations in the D-loop of gastric cancer patients ranges from 4% to 48%. The most common mutations in this region are mononucleotide repeat variants of the poly-cytosine (poly-C) sequence at nucleotide positions (np) 303-309 (D310) in mtDNA[8]. The variants were also identified in normal subjects[44] and patients with neurodegenerative diseases[45]. The effect of these variants is not clearly defined.

Moreover, several somatic point mutations identified in the mtDNA protein-coding region and tRNA genes in gastric cancer patients are potentially harmful[15]. These mutations include missense mutations (*e.g.*, G3697A and G4996A) that cause amino acid substitutions at the highly evolutionarily conserved amino acid residues, frame-shift mutations (*e.g.*, 12418insA) that result in truncated polypeptides, and tRNA mutations (*e.g.*, 7472insC) that potentially alter tRNA structure. Moreover, studies have demonstrated that these mutations are pathogenic and associated with mitochondrial diseases[15]. tRNA gene mutations as well as missense and frame-shift mutations in the mitochondrial genome may promote mitochondrial dysfunction in gastric cancer cells.

A common 4977-bp mtDNA deletion occur less frequently in gastric cancers compared with the corresponding noncancerous stomach tissues[13,46,47], though large-scale mtDNA deletions are the most common mutation in the somatic tissues of aged human subjects[9]. This finding is consistent with observations in other types of cancer[8,9]. The low accumulation of large-scale mtDNA deletions in cancer could result because an increased frequency of these mutations may cause severe mitochondrial dysfunction and sensitize the cells to apoptosis. Any cells harboring high levels of large-scale mtDNA deletions could be eliminated during tumorigenesis[9].

Unlike large-scale deletions, a 50-bp deletion flanked

Table 1 The distribution of somatic mitochondrial DNA mutations in human cancers *n* (%)

Cancer	Cases	No. of cancers with mutation	No. of mutations	D-loop	rRNA	tRNA	mRNA	Ref.
Adult leukemia	24	9 (37.5)	9	2 (22.2)	1 (11.1)	0	6 (66.7)	[16]
Bladder cancer	14	9 (64.3)	20	6 (30.0)	3 (15.0)	0	11 (55.0)	[17]
Breast cancer	18	11 (61.1)	12	7 (58.3)	0	0	5 (41.7)	[18]
	19	14 (73.7)	27	22 (81.5)	1 (3.7)	0	4 (14.8)	[19]
	15	14 (93.3)	45	17 (37.8)	3 (6.7)	2 (4.4)	23 (51.1)	[20]
	58	27 (46.6)	40	21 (52.5)	2 (5.0)	2 (5.0)	15 (37.5)	[21]
Esophageal cancer	20	11 (55.0)	14	9 (64.3)	1 (7.1)	0	4 (28.6)	[22]
Follicular thyroid cancer	3	3 (100.0)	4	2 (50.0)	2 (50.0)	0	0	[23]
Gastric cancer	31	20 (64.5)	26	18 (69.2)	0	1 (3.8)	7 (26.9)	[15]
Head-and-neck cancer	13	6 (46.2)	9	6 (66.7)	1 (11.1)	0	2 (22.2)	[17]
Hepatocellular cancer	10	5 (50.0)	24	23 (95.8)	0	0	1 (4.2)	[24]
	44	23 (52.3)	34	21 (61.8)	1 (2.9)	2 (5.9)	10 (29.4)	[25]
Lung cancer	14	6 (47.1)	10	7 (70.0)	1 (10.0)	2 (20.0)	0	[17]
	55	33 (60.0)	56	18 (32.1)	1 (1.8)	3 (5.4)	34 (60.7)	[26]
Medulloblastoma	15	6 (40.0)	18	11 (61.1)	0	3 (16.7)	4 (22.2)	[27]
Oncocytic head-and-neck tumor	25	16 (64.0)	18	0	0	0	18 (100.0)	[28]
Oncocytic pituitary adenoma	25	18 (72.0)	20	3 (15.0)	0	2 (10.0)	15 (75.0)	[28]
Oncocytic thyroid tumor	45	26 (57.8)	30	0	0	0	30 (100.0)	[29]
Oral cancer	18	14 (77.8)	26	20 (76.9)	0	0	6 (23.1)	[30]
	300	240 (80.0)	645	355 (55.0)	36 (5.6)	21 (3.3)	233 (36.1)	[31]
Ovarian cancer	10	6 (60.0)	15	11 (73.3)	3 (20.0)	0	1 (6.7)	[32]
Pancreatic cancer	5	4 (80.0)	4	0	1 (25.0)	1 (25.0)	2 (50.0)	[33]
Papillary thyroid cancer	7	3 (42.9)	4	0	0	0	4 (100.0)	[23]
Parathyroid adenoma	30	15 (50.0)	27	6 (22.2)	1 (3.3)	1 (3.3)	19 (70.4)	[34]
Renal cell cancer	8	5 (62.5)	6	1 (16.7)	2 (33.3)	0	3 (50.0)	[35]
	9	7 (77.8)	9	4 (44.4)	1 (11.1)	1 (11.1)	3 (33.3)	[36]
	15	7 (46.7)	14	4 (28.6)	4 (28.6)	1 (7.1)	5 (35.7)	[37]
Renal oncocytomas	9	9 (100.0)	14	1 (7.1)	0	0	13 (92.9)	[38]
Total	859	567 (66.0)	1180	595 (50.5)	65 (5.5)	42 (3.6)	478 (40.4)	

by a 9-bp direct repeat at nps 298-306 and 348-356 of the mtDNA D-loop region was reportedly found at high levels in four gastric cancers[48]. This deletion is associated with decreased mtDNA copy number in cancer[49].

A 260-bp tandem duplication/triplication mtDNA mutation in the D-loop region was identified in approximately 13% of the examined gastric cancers[14]. The duplicate/triplicate insertion of an 260-bp fragment is flanked by two poly-C sequences at nps 303-309 and 568-573[13,14,44]. The insertion was also detected in other types of cancer[14]. However, the occurrence of this mutation does not appear to be specific to cancer cells[14,44,50-52].

Decreased mtDNA copy number was frequently detected in gastric cancer patient tissues compared with corresponding noncancerous stomach tissue[13,53]. Alterations in mtDNA copy number change (increase or decrease) appear to be tissue specific[7,8,54]. A decreased mtDNA copy number is also found in the majority of hepatocellular carcinomas[49] and breast cancers[55].

These findings reveal that somatic point mutations and a decreased mtDNA copy number are two common mtDNA alterations in gastric cancer. The increased rate of somatic mtDNA alterations in gastric cancer is also observed in other cancers, suggesting that these two types of somatic mtDNA alterations are common events in human cancer progression. These mtDNA alterations may result from similar factor(s) and/or play a consistent role in the tumorigenesis of gastric cancer and other malignancies.

SEVERAL POTENTIAL FACTORS MAY CAUSE TO SOMATIC mtDNA ALTERATIONS IN GASTRIC CANCER

The mutation type could provide clues regarding factors that potentially contributing to somatic mtDNA alterations in gastric cancer. Among the mtDNA mutations identified in gastric cancer, 46% of the somatic point mutations are transition mutations (*e.g.*, T-to-C or G-to-A), and another 46% result from mononucleotide or dinucleotide repeat instability (*e.g.*, poly-C or poly-A)[15]. Compared with other types of cancer, 60% of the mutations are transition mutations, 31% are mononucleotide or dinucleotide repeat instability, and 4% are transversion mutations (*e.g.*, T-to-A or G-to-C) (Table 2)[15-38]. These findings indicate that transition mutations and mononucleotide or dinucleotide repeat instability are two major types of somatic mtDNA mutations in cancers.

Given that the mitochondrial electron transport chain is a major site for intracellular ROS formation, oxidative mtDNA damage is predicted to be an important factor promoting mtDNA mutations and genome instability in cancers. However, whether steady-state levels of oxidative mtDNA damage are increased in gastric cancer compared with corresponding noncancerous stomach tissue remains unknown.

The main pyrimidine and purine product of oxidative DNA base damage is thymine glycol and 7,8-dihydro-

Table 2 The types of somatic mitochondrial DNA mutations in human cancers *n* (%)

Cancer	Cases	No. of cancers with mutation (%)	No. of mutations	Transitions	Transversions	Mono-/di-nucleotide repeat instability	Others	Ref.
Adult leukemia	24	9 (37.5)	9	9 (100.0)	0	0	0	[16]
Bladder cancer	14	9 (64.3)	20	14 (70.0)	3 (15.0)	1 (5.0)	2 (10.0)	[17]
Breast cancer	18	11 (61.1)	12	6 (50.0)	1 (8.3)	5 (41.7)	0	[18]
	19	14 (73.7)	27	22 (81.5)	1 (3.7)	4 (14.8)	0	[19]
	15	14 (93.3)	45	33 (73.3)	7 (15.6)	5 (11.1)	0	[20]
	58	27 (46.6)	40	20 (50.0)	2 (5.0)	17 (27.5)	1 (2.5)	[21]
Esophageal cancer	20	11 (55.0)	14	3 (21.4)	1 (7.1)	9 (64.3)	1 (7.1)	[22]
Follicular thyroid cancer	3	3 (100.0)	4	3 (75.0)	0	1 (25.0)	0	[23]
Gastric cancer	31	20 (64.5)	26	12 (46.2)	0	12 (46.2)	2 (7.7)	[15]
Head-and-neck cancer	13	6 (46.2)	9	7 (77.8)	0	2 (22.2)	0	[17]
Hepatocellular cancer	10	5 (50.0)	24	15 (62.5)	0	9 (37.5)	0	[24]
	44	23 (52.3)	34	19 (55.9)	0	13 (38.2)	2 (5.9)	[25]
Lung cancer	14	6 (47.1)	10	8 (80.0)	1 (10.0)	1 (10.0)	0	[17]
	55	33 (60.0)	56	47 (83.9)	1 (1.8)	8 (14.3)	0	[26]
Medulloblastoma	15	6 (40.0)	18	13 (72.2)	0	5 (27.8)	0	[27]
Oncocytic head-and-neck tumor	25	16 (64.0)	18	13 (72.2)	1 (5.6)	1 (5.6)	3 (16.7)	[28]
Oncocytic pituitary adenoma	25	18 (72.0)	20	10 (50.0)	0	9 (45.0)	1 (5.0)	[28]
Oncocytic thyroid tumor	45	26 (57.8)	30	22 (73.3)	1 (3.3)	5 (15.2)	2 (6.7)	[29]
Oral cancer	18	14 (77.8)	26	12 (46.2)	4 (15.4)	8 (30.8)	2 (14.3)	[30]
	300	240 (80.0)	645	356 (55.2)	20 (3.1)	237 (36.7)	32 (5.0)	[31]
Ovarian cancer	10	6 (60.0)	15	10 (66.7)	0	4 (26.7)	1 (6.7)	[32]
Pancreatic cancer	5	4 (80.0)	4	3 (75.0)	1 (25.0)	0	0	[33]
Papillary thyroid cancer	7	3 (42.9)	4	4 (100.0)	0	0	0	[23]
Parathyroid adenoma	30	15 (50.0)	27	18 (66.7)	1 (3.7)	6 (22.2)	2 (7.4)	[34]
Renal cell cancer	8	5 (62.5)	6	2 (33.3)	1 (16.7)	1 (16.7)	2 (33.3)	[35]
	9	7 (77.8)	9	6 (66.7)	0	3 (33.3)	0	[36]
	15	7 (46.7)	14	13 (92.9)	1 (7.1)	0	0	[37]
Renal oncocytomas	9	9 (100.0)	14	7 (50.0)	2 (14.3)	4 (28.6)	1 (7.1)	[38]
Total	859	567 (66.0)	1180	707 (59.9)	49 (4.2)	370 (31.4)	54 (4.6)	

8-oxo-2'-deoxyguanosine (8-oxodG), respectively[56-59]. Thymine glycol is poorly mutagenic, but 8-oxodG can result in G-to-T transversion mutations during replication because unrepaired 8-oxodG can pair with adenine[60]. However, the most common mtDNA mutations in cancer are transition mutations rather than the mutational consequences specific to 8-oxodG (G-to-T transversion). Therefore, DNA lesions other than 8-oxodG could be primarily responsible for mtDNA transition mutations in cancer. Some studies indicated that oxidative lesion 8-oxodG can be efficiently repaired in mtDNA[61]. In addition, oxidative DNA damage can produce a range of base lesions, and the mutagenic potential of these lesions has not been fully elucidated[62]. In fact, some of these lesions may be responsible for ROS-mediated mtDNA mutagenesis. Moreover, reactive nitrogen species (RNS) can deaminate adenine to hypoxanthine, cytosine to uracil, and guanine to xanthine, thereby causing transition mutations[63,64]. Thus, it is possible that mtDNA transition mutations in cancer could result from the deamination of adenine, cytosine, or guanine by RNS. Alternatively, factors other than oxidative damage are primarily responsible for the formation of mtDNA mutations, such as defects in mtDNA polymerase or repair systems[61,65].

Oxidative damage could also contribute to mononucleotide or dinucleotide repeat instability in mtDNA[63]. The mononucleotide repeat in the D310 poly-C sequence of the D-loop region, the most common site of somatic mtDNA mutations in cancer, is the site most susceptible oxidative damage in mtDNA[66]. Moreover, extensive oxidative damage to the mononucleotide repeats may result in slippage and/or misincorporation of nucleotides during mtDNA replication or repair by mtDNA polymerase (POLG). Importantly, it has been reported that POLG is a target of oxidative damage[67] and frequently harbors mutations in cancerous tissues[68]. Specifically, mutations were identified in all three domains of the POLG protein, including the exonuclease domain, the linker region and the polymerase domain[63]. In addition, increased mtDNA mutations are observed in *Polg*$^{exo-/-}$ and *Polg*$^{exo+/-}$ mice[69,70]. Therefore, defects in the polymerase and repair activities of POLG might enhance the generation of mtDNA mutations and genome instability in cancer. However, whether a general defect in POLG *per se* leads to increased mutations or genome instability in the D-loop region compared with other region in the mitochondrial genome and the mechanisms governing this action remains unknown.

Some studies indicated that *Helicobacter pylori* (*H. pylori*) infection can affect mitochondrial function and impair DNA repair mechanisms, thereby inducing genetic instability of nuclear and mitochondrial DNA in gastric cells[71-73]. Therefore, *H. pylori* infection may promote mtDNA instability and contribute to gastric carcinogenesis in infected individuals.

Decreased mtDNA copy number could result from

mutations in the D-loop region. Because this region is the control site for mtDNA replication and transcription, mutations in the region could repress the rates of primer synthesis and mtDNA replication. This hypothesis is supported by the observation that decreased mtDNA copy number is associated with the mutations in the D-loop region[49].

In addition, decreased mtDNA copy number in cancer could be attributed to defects in mitochondrial biogenesis or other proteins localized to the mitochondria (*e.g.*, p53 or SIRT3). Defects or decreased expression in several factors involved in mtDNA replication and maintenance as well as mitochondrial biogenesis, such as POLG[68], peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1)[74], mitochondrial single-strand DNA binding protein (mtSSB)[74], and mitochondrial transcription factor A (mtTFA)[74], have been observed in cancer. Decreased mtDNA copy number correlates with reduced expression of PGC-1 in HCC[74] and mtTFA in colorectal cancer[75]. These findings suggest that reduced mitochondrial biogenesis may lead to decreased mtDNA copy number in cancers. Moreover, the tumor suppressor p53 can localize to mitochondria, and contribute to the maintenance of mtDNA stability through interactions with POLG[76]. Thus, the loss of p53 in cancer may lead to decreased mtDNA copy number. In addition, the mitochondrial deacetylase SIRT3 is down-regulated and acts as a tumor suppressor in several cancers, including gastric cancer[77-79]. The loss of SIRT3 expression is an independent prognostic marker for reduced disease-free survival and overall survival in gastric cancer[78,79]. The loss of SIRT3 is correlated with decreased mtDNA integrity and mtDNA copy number[77].

Therefore, enhanced mtDNA damages and/or reduced efficiency in the mtDNA replication and repair activities as well as the loss of mitochondrial-localized proteins may contribute to mtDNA somatic mutations and decreased copy number in gastric cancer.

CLINICAL CORRELATIONS OF SOMATIC mtDNA ALTERATIONS IN GASTRIC CANCERS

To understand the roles of somatic mtDNA alterations in gastric cancer progression, the analysis of the clinicopathological features of cancers harboring these mutations may provide insight.

We analyzed the relationships between each somatic mtDNA mutation and the clinicopathological features of gastric cancer. However, no significant correlation was observed between the clinicopathological features of gastric cancer and somatic point mutations in the D-loop[13,43] or the mitochondrial genome[15], the 4977-bp deletion[13], or the tandem duplication/triplication of mtDNA[14].

For mutations of a specific mononucleotide repeat (D310) of mtDNA, the mutations are not associated with nuclear microsatellite instability in gastric cancer[80], and are more frequent in gastric cancer patients with *H. pylori*-associated chronic gastritis compared with cancer-free patients[81]. These findings suggest that mtDNA mononucleotide instability may be involved in the early stages of gastric carcinogenesis.

A significant association between decreased mtDNA copy number and ill-defined gastric cancers, including the ill-defined ulcerative and infiltrating (Borrmann's type Ⅲ) and diffusely infiltrating (Borrmann's type Ⅳ) types, was observed[13]. A recent report further confirmed that mtDNA copy number is significantly decreased in gastric cancer, particularly in ill-defined stage Ⅲ and Ⅳ cases, and suggested that alterations in mtDNA copy number may correlate with DNA methylation[53]. Because most patients with Borrmann's type Ⅲ and Ⅳ gastric cancer have a poorer prognosis and reduced 5-year survival rate after gastrectomy, these findings suggest that decreased mtDNA copy number may modify gastric cancer progression.

THE POTENTIAL ROLES OF mtDNA MUTATIONS AND MITOCHONDRIAL DYSFUNCTION IN GASTRIC CANCER PROGRESSION

In gastric cancer, somatic point mutations in the mitochondrial coding region are potentially harmful mutations that may cause mitochondrial dysfunction. These harmful mtDNA mutations along with decreased mtDNA copy number contribute to mitochondrial dysfunction. In addition, decreased mitochondrial aconitase (ACO2) expression, decreased respiratory capacity, and mitochondrial complex I deficiency were observed in gastric cancer[82,83]. These findings have been suggested as a mechanism to explain the Warburg effect. However, the role of mtDNA mutations and mitochondrial dysfunction in tumorigenesis and cancer progression remains unclear in gastric cancer.

Among the mtDNA mutations identified in gastric cancers, the role of the 12418insA mutation in tumorigenesis has been examined using a cybrid cell model (though not in gastric cancer cells)[84]. The 12418insA mutation is an "A" nucleotide insertion in the mononucleotide repeat of a poly-adenosine (poly-A) sequence at np 12418-12425 in mtDNA. The mutation causes a frameshift and premature termination of the ND5 gene, thereby resulting in a truncated ND5 subunit protein. In addition to gastric cancer[15], this mutation was also reported in the rotenone-resistant VA2B cell line[85], colorectal cancer[86], HCC[25], and breast cancer specimens[21]. A study revealed that the heteroplasmic 12418insA mutation contributes to reduced oxidative phosphorylation and increased ROS production in human cancer cells and promotes tumorigenesis in nude mice[84]. The report provided evidence suggesting that mtDNA mutation and mitochondrial dysfunction contribute to tumorigenesis.

Additional evidence was obtained from an approach

using mitochondrial specific inhibitors to suggest that mitochondrial dysfunction enhances chemo-resistance and cell migration in human gastric cancer cells[15,87]. Oligomycin-induced mitochondrial dysfunction promotes cisplatin resistance and enhances cell migration in a human gastric cancer cell line[15]. Moreover, mitochondrial inhibitors (antimycin A and oligomycin) increased intracellular ROS levels, and the antioxidant N-acetyl-cysteine preventes the enhanced cell migration mediated by the mitochondrial inhibitors. These results suggest that ROS generated by defective mitochondria may be involved in the mechanism[15,87]. In addition, the mitochondrial inhibitors increase the expression of the cell adhesion molecule alpha5-integrin *via* ROS induction[87]. Alpha5-integrin on the cell surface is required for mitochondrial dysfunction-enhanced cell migration[87]. These findings suggest that ROS-mediated increased alpha5-integrin expression might serve as the molecular basis by which mitochondrial dysfunction promotes gastric cancer cell migration.

An addition approach employed a method to select the subpopulation of cancer cells demonstrating enhanced migration. This study indicated that highly migratory gastric cancer cells display reduced oxygen consumption rates, increased intracellular ROS content and increased alpha5-integrin expression compared with the parental cells[87]. Importantly, the evidence from clinicopathological studies with gastric cancer specimens suggest that alpha5-integrin expression is highly correlated with gastric cancer invasion[87]. These results further support the association between mitochondrial dysfunction and cell migration in gastric cancer.

Although most of the studies were not focused on gastric cancer, data from several lines of research have substantiated the pathological role of mtDNA mutation or mitochondrial dysfunction in cancer. Using cybrid cell models, pathogenic mtDNA mutation (*e.g.*, the T8993G transversion) have been shown to promote tumor growth in nude mice by preventing apoptosis[88-90]. Moreover, it was reported that the mtDNA mutation-mediated mitochondrial dysfunction contributes to metastatic cancer phenotypes, and ROS induction is mechanistically involved[88,91]. Mitochondrial inhibitors or mtDNA depletion can induce chemo-resistance or enhance the invasive phenotypes of various cancers[92-96]. "Retrograde signaling," signaling from mitochondria to the nucleus[97,98], has been proposed to be mechanistically involved. However, the common biomolecules involved in retrograde signaling remain undefined. The detailed mechanisms by which mtDNA mutation and mitochondrial dysfunction affect gastric cancer progression require further investigations.

CONCLUSION

Several types of somatic mtDNA alterations have been identified in human gastric cancers. The point mutation and decreased mtDNA copy number are the two most common mtDNA alterations, and these alterations might result in mitochondrial dysfunction in gastric cancers. These findings provide a molecular basis for the metabolic reprogramming or the "Warburg effect" in gastric cancers. Clinical correlative analyses reveal that decreased mtDNA copy number is associated with the ill-defined ulcerated and infiltrating types as well as the diffusely infiltrating types of gastric cancer, which might correlate with poorer patient prognosis[13]. However, the presence of somatic mtDNA point mutations in gastric cancers does not correlate with tumor size and grade, or patient survival[15]. This finding might be attributed to the possibility that these mtDNA point mutations do not always affect mitochondrial function nor contribute to gastric cancer progression. In addition, different heteroplasmic levels of the same mtDNA mutation might produce varying results for tumorigenesis and cancer progression. The results are consistent with *in vitro* studies using mitochondrial inhibitors, suggesting that mitochondrial dysfunction might induce chemo-resistance and enhance cell migration in part in gastric cancer cells[15,84]. Thus, the role of specific mtDNA point mutation in mitochondrial function and gastric cancer progression warrants further study.

Among the somatic mtDNA mutations identified in gastric cancer, transition mutations and mononucleotide or dinucleotide repeat instability, not transversion mutations, are the two most common types of mutation. Transition mutations may not result from oxidative DNA damage; rather, these mutations may result from specific types of DNA damage and/or reduced efficiency in mtDNA replication and repair activities as well as other undefined mechanisms.

Increasing lines of evidence have important implications in the pathological role of mtDNA mutation or mitochondrial dysfunction in gastric cancer. Increased ROS production induced by mitochondrial dysfunction may be involved in the malignant changes of gastric cancer. However, the detailed mechanism by which mtDNA mutation and mitochondrial dysfunction affect gastric cancer progression remains unclear. Elucidation of the factors causing mtDNA mutations and activating retrograde signaling pathways in gastric cancer will be important for understanding the role of mitochondria and mtDNA in gastric cancer. The search for strategies to prevent mtDNA alterations and inhibit these pathways will aid in the development of novel treatments for gastric cancers.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

New insights into the functions and localization of the homeotic gene *CDX2* in gastric cancer

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Abstract

Gastric cancer is one of the most frequent cancers, and it ranks the third most common cancer in China. The most recently caudal-related homeobox transcription factor 2 (CDX2) is expressed in a large number of human gastrointestinal cancers. In addition, gastric epithelial cell mutations in CDX2 result in tumor promotion, which is characterized by cellular drug resistance and a high proclivity for developing cancer. A series of publications over the past years suggests a mechanism by which CDX2 overexpression results in multidrug resistance. CDX2 appears to forward control regenerating IV and the multidrug resistance 1 expression signaling pathway for regulation of cell drug resistance.

Key words: Caudal-related homeobox transcription factor 2; Gastric cancer; Intestinal metaplasia; Apoptosis; Drug resistance

Core tip: This review elucidates the relationship between caudal-related homeobox transcription factor 2 (CDX2) and gastric carcinoma, and promotes research to establish whether CDX2 induces drug resistance in gastric cancer. The review highlights that CDX2-positive expression should be a useful maker for diagnosis for patients with intestinal-phenotype gastric cancer, because of this useful maker, future drug and gene therapy targets in gastric cancer might be influenced.

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INTRODUCTION

Caudal-related homeobox transcription factor 2 (CDX2) is a member of the caudal type homeobox gene family. The encoded protein is a major regulator of intestine-specific genes and is involved in cell growth and differentiation, but also has several other functions, including early embryonic development of the intestinal tract, and intestinal inflammation and tumorigenesis[1-3]. We showed that multidrug resistance was reversed in gastric cancer SGC7901/DDP cells *in vitro* and *in vivo* by CDX2 down-

regulation[4]. Overexpression of CDX2 in HT-29 cells revealed increased resistance to the known substrates of multidrug resistance protein (MDR1), vincristine and paclitaxel, which was reversed by MDR1 inhibitor verapamil[5], thereby supporting cell growth. However, high expression of CDX2 significantly reduces tumorigenicity in BGC-823 cells[6], and CDX2 may play a growth-suppressive or proapoptotic role in gastric cancer cells. These findings suggest that a unique feature of *CDX2* gene is that it plays opposing functions with regard to the regulation of cell growth and death in gastric cancer. However, the molecular networks connecting CDX2 to its function and regulation in gastric cancer remain largely unknown.

IDENTIFICATION OF CDX2

Several studies have demonstrated that CDX2 is largely present in intestinal homeostasis and inflammation[7,8]. The first description of the caudal homeobox gene was in *Drosophila* by Mlodzik *et al*[9]. Six years later, James and Kazenwadel[10] reported *CDX2* gene expression in the intestinal epithelium of adult mice. They found that all nine homeobox genes were expressed in different regions of the intestine, with a unique expression profile for each gene, and CDX2 was present in a single copy in the mouse genome. Suh and Traber[11] further showed that the intestine-specific homeobox gene, CDX2, was a transcription factor that regulated both proliferation and differentiation in intestinal epithelial cells. Rao *et al*[12] showed that overexpression of CDX2 in intestinal epithelial cells increased migration in wound healing, while a more recent work of Gross *et al*[13] indicated that decreased CDX2 expression enhanced intestinal cell migration. A similar phenomenon also occurred in gastric cancer (GC). Silberg *et al*[14] showed that ectopic expression of CDX2 induced gastric intestinal metaplasia in transgenic mice. Our recent research[15] found that overexpression of CDX2 inhibited cell growth and proliferation, blocked entry into the cell cycle S phase, reduced motility and invasion of MGC-803 cells, and increased the rate of apoptosis in GC cells *in vitro*. Moreover, Dang *et al*[16] found that loss of CDX2 predominantly altered the expression of genes involved in intestinal glandular differentiation and adhesion, but disruption of CDX2 in MKN45 cells did not significantly affect their tumorigenic potential.

CDX2 contains two conserved protein domains that play different roles. The Caudal-like protein activation region is thought to mediate transcription activation, which consists of the N termini of proteins belonging to the caudal-related homeobox protein family. The level of activation caused by mouse CDX2 is affected by phosphorylation at serine 60 *via* the mitogen-activated protein kinase pathway[17]. In this region, *CDX2* gene always has homeodomains that interact with the DNA-binding domain of DNA replication-related element binding factor, which is an 80-kDa polypeptide homodimer that plays an important role in regulating cell-proliferation-related genes[18]. Another conserved protein domain is the protein kinase and catalytic domain, which contains the catalytic domain of the serine/threonine kinase (STK), mitogen-activated protein kinase (MAPK)/MAK/MRK overlapping kinase. The protein kinase superfamily is mainly composed of the catalytic domains of serine/threonine-specific and tyrosine-specific protein kinases. It also includes the RIO kinases, which are atypical serine protein kinases, aminoglycoside phosphotransferases, and choline kinases[19]. When the catalytic domain of STKs is activated, these proteins catalyze the transfer of the γ-phosphoryl group from ATP to hydroxyl groups in specific substrates such as serine, threonine, or tyrosine residues of proteins[20]. Duncan *et al*[21] have reported that protein kinase and caspase networks induce alterations in cell survival and frequently accompany transformation and tumorigenesis.

Subsequent studies have shown that CDX2 controls the transcription of cellular genes that are essential for gastric intestinal metaplasia. CDX2 contains catalytic domain of MAPK, which is involved in various key cellular activities. And the MAPK signaling pathways have been implicated in the pathogenesis of cancer, which plays a key role in several steps of tumorigenesis including cancer cell proliferation, migration, and invasion[22]. Cell cycle progression is related to mutable transcription factors and cofactors. Several studies have shown that CDX2 is modified post-translationally, which seems to regulate its activity and modulate its interactions with other transcription factors and cofactors[17,23].

ROLE OF CDX2 IN GASTRIC INTESTINAL METAPLASIA

Gastric intestinal metaplasia is a multifocal regenerative lesion characterized by the presence of intestinal cell types, such as goblet, Paneth and absorptive cells, alone or in combination, within the gastric mucosa[24]. The ectopic intestinal glands are completely reorganized, with displacement of the proliferative zone from the neck region down to the base of the crypt, thus resembling the normal intestine, concomitant with alterations in the stromal sheath surrounding the metaplastic gland, which also acquires an intestinal phenotype[25]. Intestinal metaplasia is thus generally accepted as a preneoplastic lesion conferring increased risk for gastric cancer development[26], and its cause-effect relationship with *Helicobacter pylori* (*H. pylori*) infection is indisputable. However, intestinal metaplasia arises in only approximately 30% of infected individuals, from which only around 7% will develop gastric cancer[27]. Although low, these percentages acquire particular importance in countries where the prevalence of infection remains high, such as Asia[28], where approximately 75% of the population is infected. Over the past two decades, several animal models of developing intestinal metaplasia have been reported. The Mongolian gerbil model is the best for recreation of all gastric histological events following *H. pylori* infection leading to intestinal

metaplasia and ultimately gastric cancer, thus corroborating the causal role of infection in preneoplastic lesions and cancer development. Several studies show that after long-term infection these animals develop intestinal metaplastic lesions that resemble human disease[29,30], which develop into gastric adenocarcinoma.

Recently, the induction of an ectopic intestinal phenotype in the stomach has also been achieved in animal models by manipulating downstream events in the carcinogenic cascade. Two mouse cell lines have been developed to help understand the causal role of ectopic CDX2 expression in the stomach for development of extensive intestinal metaplasia[14]. In these models, CDX2 is under the control of promoters from different gastric-specific genes that are transcribed during embryonic development[14]. The promoter such as H^+/K^+-ATPase b-subunit[31], is only active postnatally. Both models display extensive intestinal metaplasia, presenting all intestinal cell types except Paneth cells, as well as several intestine-specific gene products typical of the different lineages. These two models suggest two separate pathways for metaplastic development. Expression of CDX2 during fetal development may affect the undifferentiated endodermal cells of the foregut, normally devoid of this protein, and thus interfere with determination of cell fate, resulting in the induction of intestinal rather than gastric differentiation in a subset of these cells. Conversely, fresh expression of CDX2 in differentiated parietal cells suggests cellular transdifferentiation, with loss of gastric marker expression and gain of intestinal markers.

Other mouse models have been shown or suggested to exhibit aberrant development of an intestinal phenotype in the stomach. The gastrin knockout mouse shows achlorhydria and develops intestinal metaplasia, with CDX2 expression, and gastric tumors[32,33]. Homozygous mutation of the SHP2-binding site within the interleukin (IL)-6 family receptor gp130 led to the development of two metaplastic lineages, spasmolytic polypeptide-expressing metaplasia (SPEM) and intestinal-like cells, as determined by the presence of acidic mucins and clear brush border morphology, but with no evidence of goblet cell differentiation[34]. Early stages of intestinal transformation of the fetal stomach are found in both Sonic Hedgehog homozygous null[35] and Gli3 null embryos, which lack this downstream effector of Hedgehog signaling[36], as assessed by alkaline phosphatase activity. However, these changes do not have an overall impact on gastric differentiation. Finally, intestinal differentiation with associated goblet cells and expression of CDX2 appear in subcutaneously grafted gastric cells derived from $Runx3^{-/-}$ mouse fetuses[37]. The same genotype in another mouse strain results in the loss of chief cells, SPEM, and an intestinal phenotype with CDX2 expression, without apparent inflammation and with increased malignant potential[38].

CDX2 SEEMS LIKELY AS AN ONCOGENE IN GASTRIC CANCER

CDX2 may have a unique role compared to other CDXs, showing characteristics of both an oncogene and a tumor suppressor[39-41]. Many researchers report that CDX2 is an inhibitor of cancer cell growth. Cell growth inhibition by CDX2 is associated with significant cell cycle arrest at the G_0/G_1 phase and CDX2 suppresses cell proliferation by controlling the G_1 and S checkpoints and inducing a specific block in cell cycle progression, after which the cells are not committed to complete the rest of the cell cycle. Many genes that are regulated in a cell-specific manner have CDX2-binding sites as their promoters, and in some cases CDX2 induces their expression directly. Some of these gene products play a direct regulatory role in the cell cycle, for example, Cdc2 and cyclin E[42,43]. Moreover, CDX2 was also forced to express by IL-6, tumor necrosis factor-α and IL-1β[44,45]. A further study showed that CDX2 promoter activity is increased by IL-6 in a MEK/ERK and phosphoinositide 3-kinase (PI3K)-dependent manner, and deletion of CDX2 binding sites in the promoter sequence results in loss of IL-6-induced promoter activity[46]. IL-6 increases CDX2 protein expression in gastric intestinal metaplasia cells that is sufficient to induce cell death. Enforced expression of CDX2 *in vitro* causes apoptosis in several cell types[6,47]. In addition, apoptosis induced by PTEN upregulation in gastric cancer cells has been shown to be dependent on CDX2, by triggering PI3K/Akt inactivation. Therefore, it was surprising to find that gastric expression of CDX2 alone was sufficient to induce intestinal metaplasia in mice, and that these mice represented a powerful tool to investigate the molecular mechanisms that promoted intestinal metaplasia[14]. Moreover, as gastric cancer in humans is often preceded by intestinal metaplasia, the phenotype described here strongly suggests involvement of CDX2 in the initiation of the process leading to intestinal neoplasia of the gastric mucosa. Several lines of evidence suggest that CDX2 has the potential to function as an oncogene in gastric carcinoma, promoting the proliferation of cells beyond their normal constraints[4,5].

For some time, this apoptotic activity of CDX2 was thought to be similar to that described for another cancer-related protein, c-Myc[48,49]. Elevation of c-Myc occurs in many tumors, resulting in potent growth promotion[50]. This effect of c-Myc can, however, only occur if the cell is also receiving appropriate survival signals, for example, leptin[51]. If not, deregulation of c-Myc will cause programmed cell death[52]. This model, however, does not completely hold true for CDX2 because mutants of CDX2 have been described, which although unable to promote cell cycle progression, retain the ability to induce programmed cell death[53]. In summary, it appears that *CDX2* acts as an oncogene in gastric cancer.

CDX2 INDUCES DRUG RESISTANCE IN GASTRIC CANCER

Regenerating protein (Reg) Ⅳ is a small, 17-kDa secreted C-type lectin that is expressed in normal enteric neuroendocrine cells and some goblet cells[54]. Reg Ⅳ is expressed in approximately 37% of gastric cancers and is detectable in the sera of approximately 36% of gastric cancer patients. Expression of Reg Ⅳ is a marker for prediction of resistance to 5-fluorouracil-based chemotherapy in patients with gastric cancer[55]. Oue *et al*[56] showed that endogenous CDX2 and Reg Ⅳ expression was correlated in gastric cancer cell lines and primary tissue, and gastric intestinal metaplasia. In addition, using an endoplasmic-reticulum- regulated form of CDX2 led to rapid induction of Reg Ⅳ expression after 4-hydroxytamoxifen treatment. Reporter gene assays revealed an important role for consensus CDX2 DNA binding elements in the Reg Ⅳ promoter region in its transcription, and subsequent chromatin immunoprecipitation assays showed that CDX2 bound directly to the Reg Ⅳ promoter[47]. These results indicate that CDX2 protein directly regulates Reg Ⅳ expression in gastric cancer and intestinal metaplasia of the stomach. Reg Ⅳ may exert its function *via* the epidermal growth factor receptor (EGFR) signaling pathway in gastric cancer. Overexpression or silencing of Reg Ⅳ influences the level of EGFR phosphorylation[57]. The EGFR signaling pathway plays an important role in the normal physiological function of cells, such as apoptosis, migration and differentiation. The signaling pathways downstream of EGFR are also central to the biology of gastrointestinal cancer. A major recent discovery has been that two major pathways mediate signal transduction through EGFR: the RAS/RAF/MAPK/ERK and the PI3K/AKT/PTEN/mTOR pathways[58]. Forced expression of Reg Ⅳ in gastric cancer cell lines also induces expression of the phosphorylated form of EGFR, Bcl-2, Bcl-XL, survivin, and the phosphorylated form of AKT[57]. Therefore, this indicates that CDX2 protein directly regulates Reg Ⅳ expression, and Reg Ⅳ activates the EGFR/Akt/AP-1 signaling pathway to improve the survival rate of cancer cells. The intestinal phenotype of gastric cancer frequently expresses EGFR[59], therefore, it is suggested that this Reg-Ⅳ-activated pathway plays an important role in this subtype of gastric cancer.

Besides, CDX2 also induces expression of the MDR1 gene by which CDX2 directly regulates expression of the gene through binding to elements in the promoter region[5]. In fact, it has been reported that postoperative chemotherapy is not beneficial for patients with intestinal phenotype gastric cancer[60]. Taken together, it is possible that in intestinal phenotype gastric cancer, expression (or ectopic expression) of CDX2 induces Reg Ⅳ and MDR1 expression, resulting in an increase in drug resistance.

CDX2 IS A USEFUL MAKER FOR FUTURE DRUG AND GENE THERAPY IN GASTRIC CANCER

Whether CDX2-positive expression can be considered as a prognostic factor for gastric cancer has been in dispute for a long time. Several investigators reported that CDX2 was an independent prognostic indicator for gastric carcinoma[61,62]. However, we showed that no significant correlation could be determined between CDX2 and clinicopathological parameters such as tumor size, invasion and lymph node metastasis in gastric cancer[63]. This suggests that CDX2 does not affect the progression of human gastric cancer. These conflicting results were likely due to small sample sizes. Meta-analysis has recently been applied to identify prognostic indicators in patients with malignant diseases[64,65]. Recently, we carried out a meta-analysis that is believed to be the first study to estimate systematically CDX2 expression and its relationship with clinicopathological characteristics and 5-year survival rate of gastric cancer patients. The results indicated that CDX2 overexpression was significantly associated with sex, lower clinical stage, tumor differentiation, lower rate of vascular invasion and lymph node metastasis, as well as higher 5-year survival rate[66]. Several investigators have reported that CDX2 expression is associated with specific morphological and mucin phenotypes of gastric epithelial dysplasia, and decreased progressively with advanced gastric cancer stage, suggesting a possible tumor suppressor role for CDX2[67-69]. However, sample sizes in the meta-analysis were too small, and whether CDX2-positive expression is significantly associated with good prognosis in patients with intestinal phenotype gastric cancer remains to be fully investigated in the future.

CONCLUSION

Ectopic expression of CDX2 occurs in the stomach and promotes intestinal metaplasia of the mucosal epithelial cells, which is an important early event in gastric tumor formation. In addition, CDX2-positive gastric cancer patients also have a higher 5-year survival rate than CDX2-negative patients. Therefore, CDX2 may be an important factor that affects the prognosis of gastric malignant tumors. CDX2 has attracted increasing interest because of its importance in modulating various cellular processes in cell growth or survival, differentiation and apoptosis *via* the regulation of gene expression. Even minor changes in nuclear CDX2 levels and/or its activities may have a significant effect on gene regulation, and thereby cellular responses, during disease pathogenesis and treatment. Therefore, an understanding of the regulatory mechanisms is of importance in intestinal phenotype gastric cancer. As few studies have reported the relationship be-

tween clinicopathological parameters and CDX2 in intestinal phenotype gastric cancer, large-sample clinical studies are needed. Elucidation of the CDX2/MDR1/Reg Ⅳ pathway is a potentially important advance in molecular oncology. In view of the high frequency of *CDX2* mutations in human gastric tumors, new and/or existing pharmacological agents directed against components of this pathway may have therapeutic benefit.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Clinical significance of lymph node metastasis in gastric cancer

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Abstract

Gastric cancer, one of the most common malignancies in the world, frequently reveals lymph node, peritoneum, and liver metastases. Most of gastric cancer patients present with lymph node metastasis when they were initially diagnosed or underwent surgical resection, which results in poor prognosis. Both the depth of tumor invasion and lymph node involvement are considered as the most important prognostic predictors of gastric cancer. Although extended lymphadenectomy was not considered a survival benefit procedure and was reported to be associated with high mortality and morbidity in two randomized controlled European trials, it showed significant superiority in terms of lower locoregional recurrence and disease related deaths compared to limited lymphadenectomy in a 15-year follow-up study. Almost all clinical investigators have reached a consensus that the predictive efficiency of the number of metastatic lymph nodes is far better than the extent of lymph node metastasis for the prognosis of gastric cancer worldwide, but other nodal metastatic classifications of gastric cancer have been proposed as alternatives to the number of metastatic lymph nodes for improving the predictive efficiency for patient prognosis. It is still controversial over whether the ratio between metastatic and examined lymph nodes is superior to the number of metastatic lymph nodes in prognostic evaluation of gastric cancer. Besides, the negative lymph node count has been increasingly recognized to be an important factor significantly associated with prognosis of gastric cancer.

Key words: Stomach; Neoplasm; Lymph node; Metastasis; Prognosis

Core tip: Many issues regarding lymph node metastasis in gastric cancer need to be addressed for improving prognostic evaluation. Theoretically, the appropriate classification of lymph node metastasis is able to improve the accurate prognosis of patients. However, it is still controversial over which classification of lymph node metastasis should be deemed as the most powerful predictor of prognosis. The optimal extent of lymph node dissection has been still debating for several decades in the world. The perfect lymphadenectomy can provide the abundant count of dissected lymph nodes for pathological examination, which is considered as the irreplaceable element for accurate evaluation of disease status. In addition, the negative node count should not be considered as a clinical variable without any significance in prognostic evaluation.

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INTRODUCTION

More than half of gastric cancer patients have lymph node metastasis when they are initially diagnosed or underwent surgical resection, which results in poor prognosis[1-4]. Lymph node involvement is the most important indictor for overall survival (OS) of gastric cancer patients following curative resection (R0), and the survival rates markedly decrease with the increase in the number of metastatic lymph nodes[5-10]. Furthermore, many investigators demonstrated that lymph node metastasis was an independent risk factor for gastric cancer recurrence in patients following curative resection[11,12]. The overall survival of lymph node-negative gastric cancer patients was significantly longer than that of lymph node-positive patients, and the overall recurrence rate in lymph node-negative gastric cancer patients was significant lower than that in lymph node-positive patients[13-16].

It is certain that a meticulous and deliberate classification of lymph node metastasis can provide the guarantee of accurately evaluating the prognosis of gastric cancer patients. To evaluate lymph node metastasis exactly, two main classifications of metastatic lymph nodes have been recommended to use in clinic by many investigators. The first classification was originated from Japan in 1980s, which defined the status of lymph node metastasis by the location of metastatic lymph nodes relative to the primary tumor according to the Japanese Classification of Gastric Carcinoma (JCGC)[17,18]. In 1997, the International Union Against Cancer (UICC) proposed that lymph node involvement should be classified according to the number of metastatic lymph nodes[19-21]. Many researchers reported that the classification based on the number of metastatic lymph nodes was more sensitive in prognostic evaluation of gastric cancer than that based on the location of metastatic lymph nodes[22-25]. In addition, the ratio between metastatic and dissected lymph nodes was deemed as an important prognostic indictor of gastric cancer after surgery[26]. So far, there has not been a universally accepted classification of metastatic lymph nodes for evaluating the overall survival of gastric cancer worldwide.

In view of the fact that regional lymph node metastasis usually begins in early period of gastric cancer, regional lymph node dissection should be recommended as part of curative resection. However, it is still debatable over the optimal extent of lymph node dissection during the curative resection for gastric cancer. Limited lymphadenectomy (D1 according to JCGC[23]) is limited to removing the perigastric lymph nodes, which can not guarantee accurate staging of lymph node metastasis and avoid potentially rudimental metastatic lymph nodes[27]. When limited lymphadenectomy is performed, no information is obtained on lymph nodes other than the perigastric ones. Conversely, if only the number of metastatic lymph nodes is used as a criterion, extended lymphadenectomy (D2 or D3 according to JCGC) could result in stage migration[28,29]. Better survival rates in extended lymphadenectomy may result from greater staging accuracy and improvement of stage-specific survival rates owing to stage migration. Although two randomized controlled European trials that compared limited lymphadenectomy with extended lymphadenectomy failed to show a survival benefit from extended lymphadenectomy[30], the lack of experience with the surgical procedure and with postoperative care were most obvious reasons to account for the poor outcome of extended lymphadenectomy[31,32]. Recently, researchers demonstrated that extended lymphadenectomy showed significant superiority in terms of lower locoregional recurrence and disease related deaths to limited lymphadenectomy in a 15-year follow-up study[33]. However, the rationale for extended lymphadenectomy as the conventional procedure of curative gastrectomy has not been elucidated clearly.

In this review, we will discuss the above-mentioned issues regarding lymph node metastasis in gastric cancer in detail.

LYMPH NODE METASTASIS FOR PROGNOSTIC EVALUATION IN GASTRIC CANCER

In gastric cancer, the presence or absence of lymph node metastasis is one of the most important prognostic indicators in patients following curative resection. More than 50% of gastric cancer patients have lymph node metastases at diagnosis, which lead to a 5-year survival rate < 30%[4]. Although several investigators reported that the minority of lymph node-negative gastric cancer patients had recurrence and poor survival, most investigators demonstrated that the prognosis of lymph node-negative gastric cancer patients was significantly better than that of lymph node-positive patients[34-36]. Hocbwald *et al*[14] analyzed the data of actual five-year survivors of gastric cancer, and found that the nodal status was the most powerful prognostic factor of outcome within the first five years after curative surgery for gastric cancer. They also demonstrated that the number of positive lymph nodes was the most important determinant of survival ($P < 0.001$) by using multivariate analysis. Ichikura *et al*[37] demonstrated that the median postoperative survival of patients with < 4 metastatic lymph nodes was significantly longer than that of patients with 4 or more metastatic lymph nodes. Gunji *et al*[38] also found that early gastric cancer patients with 4 or more metastatic lymph nodes had a higher probability of recurrence and shorter short-term survival. In a previous study, we also demonstrated that gastric cancer patients with positive lymph nodes had much shorter median OS than those with negative lymph nodes[39].

Recurrence is the most common reason of cancer related death in gastric cancer patients. Owing to the fact that more than half of gastric cancers was advanced at the time of diagnosis, even when the curative resection (R0) was possible, the recurrence could occur in approximately 60% of patients[40]. The recurrence of surgically resectable gastric cancer is influenced significantly by the presence of lymph node metastasis. The more the number of metastatic lymph nodes, the worse the prognosis of gastric cancer patients[41]. Besides, we found that the number of metastatic lymph nodes was the most important indicator of recurrence after curative surgery[42]. In a further analysis, we demonstrated that the locoregional recurrence of gastric cancer was significantly associated with the number of metastatic lymph nodes ($P = 0.025$)[43]. Hepatic metastasis is the most frequent distant metastasis of gastric cancer following curative resection. The detail mechanism of hepatic metastasis from gastric cancer after curative resection is unclear. Although the hematogenous metastasis of gastric cancer was deemed as the most important route of hepatic metastasis following curative resection[44,45], some researchers demonstrated that lymphatic metastasis should be another reason of hepatic metastasis[46]. In our previous study, we demonstrated that lymph node metastasis was not only the most important factor of hepatic metastasis of gastric cancer following curative resection, but also the independent factor of the interval time between radical gastrectomy and hepatic metastasis[47]. Unlike in the West, peritoneal dissemination is the main pattern of recurrence after curative gastrectomy in the East[48]. We also demonstrated that peritoneal dissemination was the most usual recurrence type of gastric cancer after surgery[43]. Meanwhile, we found that the number of metastatic lymph nodes was significantly associated with the occurrence of peritoneal dissemination.

THE OPTIMAL CLASSIFICATION OF LYMPH NODE METASTASIS FOR PROGNOSIC EVALUATION IN GASTRIC CANCER

The first classification of metastatic lymph nodes was originated from Japan in 1980s, which defined the status of lymph node metastasis by the location of positive lymph nodes relative to the primary tumor according to the JCGC. Once extragastric lymph node metastasis is identified, the probability of systemic cancer dissemination significantly increases in theory. In our latest study, we demonstrated that extragastric lymph node was an important factor associated with the dismal prognosis of patients, even those undergoing extended lymphadenectomy[49]. However, many researchers reported that classification based on the number of metastatic lymph nodes was more sensitive in prognostic evaluation of gastric cancer than that based on the location of metastatic lymph nodes. Although the JCGC classification of lymph node metastasis can reflect the metastatic pathways of cancer cells from the primary tumor, the non-quantitative characteristics of this classification lead to inefficient evaluation of the prognosis of gastric cancer patients. As we know, comparatively accurate results of data statistics should be based on the continuous data or the elaborate classification of non-continuous data. Therefore, the UICC has still maintained the classification of lymph node metastasis based on the number of lymph node metastasis (the $5^{th}/6^{th}$ N stage) to provide the accurate prognostic evaluation for gastric cancer patients since 1997. However, it is still controversial over the best cut-offs of the number of metastatic lymph nodes for prognostic evaluation. So many researchers proposed that the N staging should be updated for improvement of its accuracy for prognostic evaluation in gastric cancer in clinical application[41,50]. Although the $5^{th}/6^{th}$ edition UICC TNM node staging system is deemed to have higher feasibility, objectivity, reproducibility, and increased strength of prognostic stratification than other edition node staging systems, it has resulted in inevitable controversies ultimately. There has not been a universally accepted node staging system for extremely precise evaluation of the relationship between the $5^{th}/6^{th}$ edition UICC N stage and prognosis of gastric cancer patients[50-52]. In 2010, we identified that the 7^{th} edition UICC N stage was superior to the $5^{th}/6^{th}$ UICC N stage and Japanese n stage for prognostic evaluation in gastric cancer patients[53]. Subsequently, many researchers reported that the 7^{th} edition UICC N stage could provide a more stratified survival difference in sub-staged gastric cancer than the $5^{th}/6^{th}$ edition UICC N stage, which should be considered to be much more reasonable compared with the previous edition N stage, especially between the N1- and N2-stage tumors[54-57]. On the other hand, a few authors did not recommend that the 7^{th} edition UICC N stage as the optimal classification of lymph node metastasis in gastric cancer[58,59].

Many studies indicated that the ratio between metastatic and dissected lymph nodes, namely the number of metastatic lymph nodes to that of dissected lymph nodes, was an independent prognostic factor for gastric cancer and other malignant neoplasms, and was reported to be able to reduce the phenomenon of stage migration[60,61]. Nevertheless, the prognostic superiority of the ratio has been still controversial for many years[62]. In the previous study, we demonstrated that the ratio between metastatic and dissected lymph nodes was inferior to the N stage for predicting the OS of gastric cancer patients with 15 or more dissected lymph nodes after curative resection[50]. Kulig *et al*[63] demonstrated that the ratio between metastatic and dissected lymph nodes could not be regarded as a standard classification of lymph node metastasis alternative to other classifications after curative gastrectomy plus extended lymphadenectomy. Actually, the ratio between metastatic and dissected lymph nodes can not define the number of examined lymph nodes and formulate the extent of lymph node dissection, which is considered the potentially key factor interfering with the

quality of curative surgery and the prognosis of patients. We also demonstrated that the ratio between metastatic and dissected lymph nodes could enhance the prognostic evaluation accuracy of the N stage, although it was identified to be inferior to the N stage for accurate evaluation of patient prognosis[42].

THE APPROPRIATE EXTENT OF LYMPH NODE DISSECTION

Lymph node metastasis can occur during the early stages of gastric cancer, and regional lymphadenectomy is recommended as part of radical gastrectomy. However, the extent of lymphadenectomy to achieve the optimal result is controversial, and there is no worldwide consensus. Once extragastric lymph node metastasis of gastric cancer is identified[49], the probability of systemic dissemination of tumor cells will significantly increase. The optimal extent of lymph node dissection is an unavoidable problem which can interfere with the accurate evaluation of the extent of lymph node metastasis. Primary tumors were conventionally resected en bloc with limited or extended lymphadenectomy (D1 or D2-3 according to the JCGC). The limited lymphadenectomy (D1) entails the removal of the perigastric nodes only, whereas extended lymphadenectomy (D2 or D3) involves the removal of both perigastric and extragastric nodes. Extended lymphadenectomy can afford more exhaustive information of nodal metastasis than limited lymphadenectomy theoretically, while it has not reached a consensus of the standard extent of lymph node dissection for gastric cancer. Early studies have reported that 30%-40% of patients with metastatic lymph nodes including the second tier lymph nodes have survived longer than 5 years after extended lymphadenectomy[64]. Although extended lymphadenectomy was not considered a survival benefit procedure as it was reported in two randomized controlled European trials[65,66], the lack of experience with surgical procedure and with postoperative care was thought to account for the poor outcome of patients who underwent extended lymphadenectomy[67-69]. Ultimately, extended lymphadenectomy showed significant superiority in terms of lower locoregional recurrence and disease related deaths to limited lymphadenectomy in the 15-year follow-up study[33]. Recently, many Western clinical researchers also demonstrated that extended lymphadenectomy could improve survival and decrease the perioperative morbidity of gastric cancer patients[70-72].

Nodal metastatic stage redefinition and local relapse decrease were deemed as the most important reasons for improvement in the prognosis of gastric cancer patients who underwent extended lymphadenectomy, even in patients with node-negative gastric cancer[73,74]. Theoretically, extended lymphadenectomy allows to harvest more lymph nodes and eliminate more broadly perigastric lymphatic tissues which were potentially invaded by tumor cells, compared with limited lymphadenectomy[75]. The extent of surgery will especially influence locoregional control. In a Dutch trial, locoregional recurrence was registered in 58% of cases in the limited lymphadenectomy group and in 45% of cases in the extended lymphadenectomy group. However, the Japan clinical oncology group 9206-1 trial demonstrated that local relapse rate was only < 1% following curative gastrectomy with extended lymphadenectomy[76]. Yoshikawa *et al*[77] analyzed 1041 early gastric cancer patients who underwent extended lymphadenectomy with curative intent, and found that 15 cases had died of recurrence (1.44%) at the last follow-up.

Although high morbidity and mortality rates have been reported in patients requiring extensive gastric resection[78], extended lymphadenectomy has a much lower morbidity and mortality in Japan than in the West, with a mortality rate being less than 3%[79]. Biffi *et al*[80] analyzed 250 gastric cancer patients in a single center and demonstrated that extended gastrectomy with spleen and pancreas routine preservation can be considered a safe treatment for gastric cancer in Western patients, at least in experienced centers. In the Japan clinical oncology group study 9501, the reported hospital mortality was only 0.80%, which is significantly lower compared with Western reports[81]. Recently, an Italy randomized clinical trial demonstrated that extended lymphadenectomy could be considered a safe option, with a mortality of only 3.0% in Western gastric cancer patients[82]. Wu *et al*[67] reported that extended lymphadenectomy was a sufficiently safe procedure for gastric cancer patients, even without hospital morbidity.

THE MINIMUM NUMBER OF LYMPH NODE DISSECTED

In 1997 the UICC/American Joint Committee on Cancer (AJCC) recommended, in the 5th edition of the staging manual, that a minimum of 15 lymph nodes need to be assessed per patient[83]. However, the 7th edition TNM classification of N stages of gastric cancer states that "histological examination of a regional lymphadenectomy specimen will ordinarily include 16 or more lymph nodes". In this classification, it is only a recommendation and no longer a prerequisite that there is to be no less than 16 dissected lymph nodes for adequate N stage classification. Of note, the 7th edition N staging does specify that at least 16 metastatic lymph nodes are required to assign N3b stage to gastric cancer patients[84]. Studies have shown that stage migration occurs in patients with a lower number of lymph nodes examined, creating inaccuracies in survival prediction[85-87]. Smith *et al*[31] proposed that the total lymph node number analyzed is an important and powerful qualifier of staging information and survival evaluation for gastric cancer, regardless of the underlying mechanisms that influence this survival impact of lymph node counts. They demonstrated that the trend toward superior survival outcome could be followed to lymph node counts greater than 40, although there was no unambiguous cutoff point for numerical lymph node analy-

sis after curative gastrectomy.

Theoretically, a consensus should be reached that inadequate lymph node assessment directly affects patient survival[88-90]. Stage migration was occasionally identified to arise in patients with even great number of dissected lymph nodes, although many researchers demonstrated that the effect of stage migration is most usually shown in patients with a small number of lymph nodes examined[31,91]. It is certain that lymph node counts reflect not only the actual number of lymph nodes removed intraoperatively but also (or especially) the number of lymph nodes identified and properly examined during macroscopic and microscopic pathologic analysis[92]. Several investigators reported that the more the number of lymph node dissection, the longer the disease-free survival (DFS) of gastric cancer patients[89,90]. Besides, there was a significantly negative correlation between the number of dissected lymph nodes and the local recurrence rate in patients after curative gastrectomy in above-mentioned studies. In the previous study, we have demonstrated that patients presented with no less than 15 dissected lymph nodes had comparatively longer median OS after recurrence, compared to those with less than 15 dissected lymph nodes[90]. Scartozzi *et al*[91] reported a remarkable decrease in local recurrence rate, from 23% in patients with less than 25 lymph nodes assessed to 4.7% in patients with no less than 25 lymph nodes assessed. They considered that abundant lymph node dissection should be associated with good locoregional control. Another example, in the 15-year analysis of the Dutch D1/D2 randomized controlled trial, local recurrence was 22% in the D1 group, compared with 12% in the D2 group; while regional recurrence was 19% in the D1 group, and 13% in the D2 group ($P = 0.015$)[33]. In 2012, Xu *et al*[93] demonstrated that it is necessary to examine at least 16 lymph nodes for accurate pathological examination of gastric cancer, even in node-negative gastric cancer patients.

NEGATIVE NODES IN GASTRIC CANCER

Negative lymph node count has been fully given importance for its significant association with the prognosis of patients with a malignant disease[94-96]. It is undoubted that patients with negative node metastasis present with a higher 5-year survival rate (5-YSR) and lower recurrence rate than those with positive node metastasis after curative resection for gastric cancer[97]. Owing to the comparatively better bio-behaviors of tumor, gastric cancer patients who present with no nodal involvement can have a higher 5-YSR regardless of lymphadenectomy[98]. Besides, the negative lymph node count is significantly associated with the prognosis of patients. Theoretically, the negative lymph node count should be deemed as the important guarantee for real R0 resection of gastric cancer and significantly associated with the OS of gastric cancer patients owing to micro-metastasis in negative nodes which could not be identified by conventional pathological examination[99,100]. It is so crucial that the total harvested lymph nodes must comprise negative lymph nodes, which is the basic guarantee for really radical resection of gastric cancer without leaving over any potential metastasis theoretically. By increasing the number of negative lymph nodes evaluated, the chance of leaving the micrometastasis within negative lymph nodes decreases.

Recent data demonstrate that the immunohistochemical evaluation of histologically negative lymph nodes detects micro-metastatic disease with varying frequencies even in early gastric cancer. Kim *et al*[99] demonstrated that the incidence of lymph node micro-metastasis in early gastric cancer patients with negative lymph nodes was less than 10%. Saito *et al*[100] demonstrated that the micrometastases within lymph nodes which were determined by using the immunohistochemical anti-cytokeratin antibody in node-negative early gastric cancer patients were closely associated with recurrence following curative gastrectomy. Endo *et al*[101] used anti-cytokeratin antibody to immunohistochemically detect nodal micrometastasis that was not identified by routine pathological examination in 162 patients with apparent node-negative submucosal gastric cancer. The micrometastasis was detected in 45 of 2048 nodes (2.2%), representing 31 of 162 patients (19%). Although the micro-metastasis and isolated tumor cells in negative lymph nodes are considered as the key factors that could lead to an adverse effect on the OS of patients, patients with negative lymph node metastasis who were identified to have isolated tumor cells or micro-metastasis did not demonstrate a significantly worse prognosis than those who did not have isolated tumor cells after curative gastrectomy with extended lymphadenectomy[102]. Harrison *et al*[103] demonstrated that T3N0M0 gastric cancer patients who underwent extended lymphadenectomy had significantly more negative lymph nodes than those who underwent limited lymphadenectomy, indicating that extended lymphadenectomy can improve the OS of T3N0M0 patients. This result is potentially associated with the elimination of micro-metastasis in negative lymph nodes.

In a previous study, we also found that the negative lymph node count was an independent predictor of OS in gastric cancer patients with perigastric node metastasis, as was the positive lymph node count[104]. Further analysis demonstrated that the negative node count was the most intensive predictor of OS in patients with perigastric lymph node involvement after extended lymphadenectomy. Ultimately, we deduced that extended lymphadenectomy improved the prognosis of patients with perigastric lymph node involvement possibly by dissecting more negative nodes, compared to the limited lymphadenectomy. Recently, the sentinel node navigation was demonstrated to be an important factor influencing the diagnosis and treatment of lymph node metastasis from gastric cancer, especially in early stage patients[105]. Clinical application of the sentinel node navigation in gastric cancer is still restricted to early gastric cancer patients, owing to the complexity of the lymphatic drainage pathways of

the stomach. The results of two noted random control trails (JCOG0302 and SNNS) of the sentinel node navigation in Japan failed to demonstrate that the detection of sentinel lymph nodes could exactly predict the status of lymph node metastasis intraoperatively, which implied that lymphadenectomy should be irreplaceable for the elimination of nodal metastasis[106].

CONCLUSION

Lymph node metastasis, as one of the most intensively prognostic indictor of gastric cancer, needs to be further investigated to elucidate the optimal significance for improving the prognostic evaluation and clinical treatment. Although the number of metastatic lymph nodes is considered the most appropriate classification of nodal metastasis for evaluation of OS, the negative lymph node count is an important variable that can provide important information contributing to prognostic evaluation and clinical treatment of gastric cancer. In addition, the extent of lymphadenectomy should be deeply investigated by studying the basic metastatic mechanism of cancer cells.

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REVIEW

Post-infectious irritable bowel syndrome: Mechanistic insights into chronic disturbances following enteric infection

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Abstract

Irritable bowel syndrome (IBS) is a commonly encountered chronic functional gastrointestinal (GI) disorder. Approximately 10% of IBS patients can trace the onset of their symptoms to a previous a bout of infectious dysentery. The appearance of new IBS symptoms following an infectious event is defined as post-infectious-IBS. Indeed, with the World Health Organization estimating between 2 and 4 billion cases annually, infectious diarrheal disease represents an incredible international healthcare burden. Additionally, compounding evidence suggests many commonly encountered enteropathogens as unique triggers behind IBS symptom generation and underlying pathophysiological features. A growing body of work provides evidence supporting a role for pathogen-mediated modifications in the resident intestinal microbiota, epithelial barrier integrity, effector cell functions, and innate and adaptive immune features, all proposed physiological manifestations that can underlie GI abnormalities in IBS. Enteric pathogens must employ a vast array of machinery to evade host protective immune mechanisms, and illicit successful infections. Consequently, the impact of infectious events on host physiology can be multidimensional in terms of anatomical location, functional scope, and duration. This review offers a unique discussion of the mechanisms employed by many commonly encountered enteric pathogens that cause acute disease, but may also lead to the establishment of chronic GI dysfunction compatible with IBS.

Key words: Post-infectious irritable bowel syndrome; Infectious diarrhea; Enteric pathogen; Inflammatory disorders; Immune alterations

Core tip: This review discusses the long-term consequences of acute enteric infections that may serve to trigger post-infectious irritable bowel syndrome, a routinely diagnosed disorder. This unique discussion elucidates novel initiation mechanisms, underlying pathophysiological features of post-infectious irritable bowel syndrome, employed by commonly encountered enteric pathogens.

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INTRODUCTION

Irritable bowel syndrome (IBS) is among the most commonly encountered chronic functional gastrointestinal (GI) disorders afflicting individuals in westernized nations. Based on the Rome Ⅲ criteria abdominal pain accompanied by sustained changes in bowel habit constitute IBS, whose diagnosis is achieved in the absence of

biochemical markers of disease[1]. Clinical presentation of constipation, diarrhea, or a combination, constitutes the different subtypes of IBS: IBS with constipation (IBS-C), diarrheal IBS subtype (IBS-D), mixed IBS (IBS-M), respectively[2]. Often perceived as a female-dominant disorder, IBS is thought to afflict between 5%-10% of the population[3], especially in westernized nations. Elucidating the mechanisms underlying the typical multifaceted clinical presentation of IBS is a topic of considerable research efforts in the medical community[4]. A growing body of evidence implicates numerous triggering events in contributing to IBS pathophysiology, including an initiating bout of infectious enteritis, low grade inflammation, altered functionalities in GI cell types, increases in epithelial permeability, and alterations in the GI microbiota, although the precise mechanisms of underlying each remain obscure[2,5-8]. Approximately 10% of IBS patients believe that their symptoms began following a bout of infectious dysentery[6], leading to the coinage of the term; Post infectious (Pi)-IBS. While many enteric pathogens cause self-limiting, acute diarrheal disease, subsequent chronic physiological consequences may persist in some individuals[9]. Many commonly encountered enteric pathogens can produce physiological changes that may provide important initiation mechanisms underlying chronic GI conditions, such as Pi-IBS. This article critically reviews the evidence supporting a role for key physiological changes initiated during enteric infection, that may in turn be responsible for IBS symptom.

Pi-IBS

Based on the Rome criteria for diagnosis, any onset of new IBS symptoms subsequently following an infectious event is defined as Pi-IBS[6]. Pi-IBS cases often exhibit characteristics of the IBS-D, and can occur in 4%-31% of patients following acute gastroenteritis[6,10-12]. A large body of work provides evidence supporting a role for pathogen-mediated modifications in the resident intestinal microbiota, epithelial barrier integrity, enterochromaffin cell function, and innate immune features[5,13,14] in Pi-IBS manifestation. Any number of these pathogenic consequences have been reported following enteric infection incited by an array of pathogens such as *Shigella* spp., pathogenic *Escherichia coli*, *Salmonella*, *Campylobacter jejuni*, and *Giardia duodenalis*[14-18]. Enteric pathogens must employ a vast array of machinery to evade the host protective immune mechanisms, and illicit successful infections. Recent work identifying genetic mutations, namely in genes responsible for epithelial and innate immune functionalities, in patients experiencing both the post-infectious, and traditional forms of IBS, point to defects in innate immunity and epithelial homeostasis as an important risk factor for IBS susceptibility[19,20]. The impact of infectious events on host physiology can be multidimensional in terms of anatomical location, functional scope, and duration. Indeed, anatomical, immunological, and neurological dysfunctions, or combinations of such, have all been shown as risk factors determining Pi-IBS manifestation. This review will provide an in-depth discussion surrounding the potential roles in which a variety of commonly encountered enteric pathogens may play in initiating important pathophysiological features of Pi-IBS.

CLINICAL PRESENTATIONS OF IBS FOLLOWING ENTERIC INFECTION: ALTERED INTESTINAL MOTILITY AND HYPERSENSITIVITY

Abnormal bowel habits and abdominal hypersensitivity, or reduced threshold of pain, are the hallmark clinical signs of IBS. The classification of IBS as a functional disorder stems from a lack of determinant histopathological, or structural biomarkers in afflicted patients. The Rome criteria requires the incidence of abdominal pain, accompanied by alterations in bowel habit for complete IBS diagnosis[21].

Altered intestinal motility

Abnormal GI motility is commonly associated with altered bowel habits producing diarrheal, constipation, and mixed IBS subtypes[22]. The potential for dysfunctional intestinal motility in contributing to altered bowel habits in IBS is supported by studies looking at intestinal transit rates between healthy and IBS individuals, with IBS-D subtypes exhibiting enhanced rates of SI transit, and the opposite trend observed for IBS-C patients[22,23]. Moreover, a recent report demonstrated that the normal colorectal reflex (normal increase in rectal tone in response to phasic colonic distention) was largely abolished in IBS patients, regardless of bowel habit, providing some evidence for altered colonic motility in these individuals[24]. Interestingly, muscle hypercontractility and abnormal motility patterns are observed subsequent to *Trichinella spiralis* infection in a commonly used murine model of PI-IBS[13,25-27], suggesting that persistent dysfunctional intestinal motility can be incited following an acute infection.

Abdominal hypersensitivity

Lower thresholds for pain tolerance in IBS patients have been documented along the entire length of the GI tract[22], an effect that is thought to occur in upwards of 60% of afflicted individuals[7]. Hypersensitivity often occurs locally in response to colonic distention[7]. Furthermore, overall visceral hypersensitivity, even upon brief stimuli such as the ingestion of food, is well documented in IBS patients, and may contribute to additional bloating, nausea, and urgency symptoms[8,28].

Stressful events can drastically affect the processing of visceral stimuli[29,30] and result in dysfunctional central neural processes culminating into heightened pain perception. Injury to visceral afferents, for example, is a common cause underlying visceral hypersensitivity[7]. Studies using a rat model of TNBS-induced transient colonic inflammation have highlighted that persistent tis-

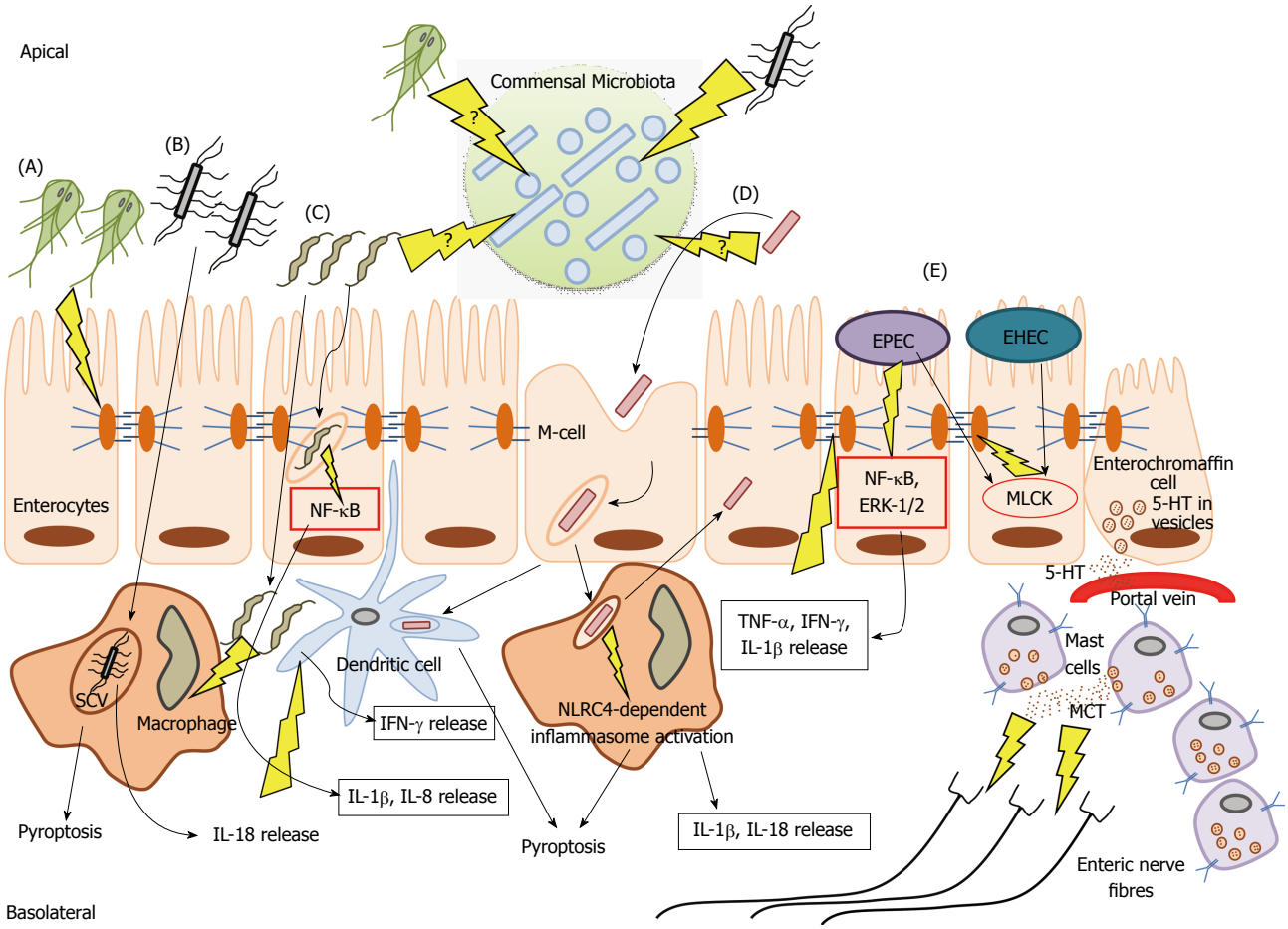

Figure 1 Illustration representing the interaction of several pathogens with the intestinal epithelium and resident immune cells, and their contribution to the development of post-infectious-irritable bowel syndrome. A: *Giardia duodenalis* disrupts tight junctional proteins in the epithelium, in addition to resulting in a decrease in 5-HT-producing enterochromaffin cells; B: *Salmonella* enterica serovar *Typhimurium* invades enterocytes and makes its way to resident macrophages, where upon being phagocytosed it causes interleukin (IL)-18 release, which further stimulates interferon (IFN)-γ release from nearby immune cells, *i.e.*, lamina propria dendritic cells, and macrophage pyroptosis. This pathogen is also able to disrupt the resident microbiota; C: *Campylobacter* jejuni causes disruptions in TLR9 signaling to make epithelial cells more susceptible (would sensitive apply here instead of susceptible?) even to mild pro-inflammatory cytokines. It also activates the NF-κB pathway to result in an IL-1β and IL-8 release. *C. jejuni* has also been shown, particularly in cases of post infectious (Pi)-IBS, to cause a reduction of resident $CD68^+$ macrophages; D: *Shigella flexneri* crosses the epithelium through the M cell and is taken up resident macrophages, where it causes IL-1β and IL-18 release, and pyroptosis in these macrophages. *S. flexneri* has also been associated with increased number of mast cells, secretions of which MCT can activate the enteric nervous system; E: EPEC results in TNF-α, IFN-γ, and IL-1β release *via* NF-κB and ERK-1/2 activation. Both EPEC and EHEC result in MLCK-dependent tight junctional disruption. Intriguingly, *G. duodenalis* (A) and *C. jejuni* (C) have been implicated in the modification of the intestinal microbiota; however, the effects of this modification remain unclear[90]. A variety of combinations of these factors may contribute to the pathogenesis of PI-IBS. MCT: Mass cell tryptase.

sue injury may directly produce heightened visceral pain perception[31]. Importantly, chemically induced colonic inflammation models have stark parallels to many of the physiological events accompanying enteric infections. Initial processes of inflammation, for instance, may act to first sensitize effector, neuronal, and immune cells within the GI tract.

Interestingly, many of the physiological consequences that can result from infectious events within the GI tract have also been proposed as determinants capable of contributing to abnormal motility and hypersensitivity symptoms seen in IBS patients. The major mechanisms currently thought to underlie IBS pathogenesis, and the evidence surrounding possible contributions made to each by distinct enteric pathogens, will be discussed in the following sections (Figure 1).

PATHOPHYSIOLOGICAL FEATURES OF IBS FOLLOWING ENTERIC INFECTION

Immune system alterations

Accumulating evidence suggests subtle alterations in the immune system in both the gut, and peripheral circulation of PI-IBS patients[32]. Pathogen-mediated disruptions of the mucosal barrier have the ability to allow for persistent immune activation within the intestine, largely due to increased exposure to luminal antigens. Likewise, the host inflammatory response towards perceived pathogens, while meant to be protective, may result in detrimental, perpetuated activation of effector cells and inflammatory mediators. The incidence of PI-IBS symptoms in many patients following enteric infection has fuelled interest in looking at persistent immune infiltrate, and/or altered

immune functionalities, as plausible driving forces in the generation of IBS symptoms[33].

Mast cells/macrophages/dendritic cells: Certain enteric pathogens have been shown to promote mast cell accumulation. A recent study found that a large proportion of patients experiencing Shigellosis, caused by invasive *Shigella* spp., go on to develop PI-IBS, and that this effect is accompanied by augmented mast cell numbers[34]. Under normal conditions, mucosal mast cells are highly involved in wound-healing, and defense against pathogens[5]. However, multiple reports document heightened numbers of mast cells within the small[35,36], and large intestines[37-39] of IBS patients. One study, which observed increased mast cells specifically within the duodenum of IBS patients suggested that infiltration of these cells may provide some explanation behind the observation that symptoms differ depending upon the affected site along the GI tract[36]. Also, mast cells can secrete serotonin, therefore increased populations of these cells may provide a link between cellular infiltrate and altered serotonin signaling leading to changes along the brain-gut axis, and dysmotility, characteristic of either IBS-D or IBS-C[36]. Furthermore, augmented numbers of mast cells, and particularly those closely associated with nerve fibers, have been reported in both IBS and Pi-IBS[38] (Figure 1), an effect which may be correlated with enhanced bloating and pain perception symptoms[2,40-42].

The *T. spiralis* mouse model of Pi-IBS has provided important insight into many pathophysiological changes following acute enteric infection. A recent study, for instance, documented numerical and phenotypic alterations in lamina propria dendritic cells (LPDC), following acute *T. spiralis* infection[43]. In what the authors defined as the "Pi-IBS stage" of infection, *i.e.*, no recovery of nematode in the stool, LPDCs exhibited enhanced expression of co-stimulatory molecules, and greater ability to migrate to and drive $CD4^+$ T cell proliferation[43]. Furthermore, the altered LPDC phenotype was proposed to underlie enhanced levels of pro-inflammatory interferon (IFN)-γ, IL-23 and tumor necrosis factor (TNF)-α production in the Pi-IBS stage[43]. The important role that these cells play in directing T-cell responses may have implications in promoting a low-grade inflammatory milieu, and requires further investigation in relation to IBS pathogenesis.

Monocytes and macrophages are at the forefront of initiating an inflammatory response to pathogens, in addition to providing essential directives to the adaptive immune system[5]. In Pi-IBS cases confirmed following *C. jejuni* infection the numbers of resident $CD68^+$ macrophages are diminished, perhaps owing to the cytotoxic nature of the pathogen inside host cells[9]. Likewise, *Shigella spp.*[15,16] and *Salmonella* infections have been implicated in causing Pi-IBS, and both are obligate intracellular pathogens, which preferentially exploit phagocytic machinery of the macrophage. Specifically, *Shigella* is transported into the lamina propria through M cells in the epithelium, and presented to resident macrophages and dendritic cells (DCs) for phagocytosis upon which activation of the nucleotide-binding oligomerization domain (NOD)-like receptor protein (NLRC4) inflammasome occurs[44,45] (Figure 1). Consequently, the resulting activation of pro-inflammatory cytokines, interleukin (IL)-18 and IL-1β, are thought to be major determinants of the high inflammatory conditions characteristic of early *Shigella* infection[45]. Inflammasome activation can also produce heightened rates of macrophage cell death *via* pyroptosis, which acts as an "inflammatory" form of programmed cell death (Figure 1). Thus, *Shigella* infection promotes a high status of inflammation, while simultaneously resulting in the detrimental loss of lamina propria (LP) macrophages. LP macrophages have an important regulatory, and anti-inflammatory role in maintaining intestinal homeostasis[45]. Furthermore, as a consequence of resident LP macrophage depletion, additional circulating monocytes may be recruited to the site of infection, and often differentiate into macrophages possessing a more pro-inflammatory capacity[45]. Considering ample reports documenting low-grade inflammation IBS patients[46,47], pathogen-mediated inflammatory conditions, in addition to the promotion of pro-inflammatory cell phenotypes, may be especially relevant triggers underlying Pi-IBS development.

In contrast to *Shigella*, *Salmonella* is seemingly less cytotoxic to macrophages[48], yet Pi-IBS symptoms have been reported following anywhere between 6%-32% of confirmed infections[2,19]. Following phagocytosis, *Salmonella* forms the characteristic Salmonella Containing Vacuole (SCV) in macrophages, in which it replicates while effectively evading host immune machinery, and pyroptosis[48] (Figure 1). While capable of avoiding certain immune parameters, *Salmonella* still evokes a strong IL-18 response[48] which has important implications in exerting paracrine effects on surrounding immune cells to induce IFN-γ expression, and also result in increased levels of activated T cells in the infected intestine, accumulation of which has been documented in many examinations of IBS[9,32,33,42,49].

Cytokine profiles: Substantial regulation exists within the GI tract in order to maintain a functional balance between pro- and anti-inflammatory mediators under homeostatic conditions. Engagement of the Toll-like receptors (TLRs), NOD-like receptors (NLRs), and other host pathogen-recognition-receptors (PRRs) occurs through ligation by various pathogen-associated-molecular-patterns (PAMPs). *Shigella*, for instance, is known to stimulate excess production of IL-1β from immune cells during infection *via* the NLRC4 inflammasome[44,45] (Figure 1). Also, excessive IL-8 secretion is a hallmark of *Campylobacter* pathogenesis[50], and is initiated upon host recognition of the pathogen-associated lipooligosaccharide[51]. Interestingly, a recent report demonstrated a disruption in TLR9 expression on epithelial cells to be implicated in the enhanced susceptibility to mild pro-inflammatory stimuli post-campylobacteriosis in mice[52]. *C. jejuni* is also know to promote the translocation of non-invasive commensal

bacteria *via* paracellular and transcellular pathways[53,54]. *Campylobacter* has also been shown to activate copious amounts of nuclear factor (NF)-κB and IL-1β from immune cells, *in vitro*[51]. Likewise, recognition of EPEC flagellin and endotoxin results in NF-κB and extracellular signal regulated kinase (ERK)-1/2 –driven IL-8 release, and enhanced TNF-α, IFN-γ and IL-1β in the infected mucosa[55,56] (Figure 1). Interestingly, at least some of the pro-inflammatory cytokines, including TNF-α, IL-1β, and IFN-γ may themselves disrupt the epithelial barrier through alterations of the tight junctions (TJs), and promote increased permeability[57-59]. Thus, residual pro-inflammatory infiltrate following enteric infection combined with the sub-epithelial penetration of commensal bacteria, can create extensive damage to surrounding intestinal tissues, and likely promote chronic pathophysiological consequences. Consequently, many reports have drawn links between altered cytokine profiles and IBS generation[60], and findings include increased levels of pro-inflammatory IL-6, IL-8, and TNF-α in plasma and circulating blood mononuclear secretions from IBS patients[47,61]. Lower detection of typical anti-inflammatory cytokines, IL-10 and transforming growth factor (TGF)-β, at the level of mRNA has also been reported[62]. Also, evidence from the *T. spiralis* Pi-IBS murine infection model has shown greater levels of IFN-γ, IL-23 and TNF-α produced by DCs in the Pi-IBS stage[43]. Additionally, sustained levels of pro-inflammatory mediators have been documented in a 21-d *Citrobacter rodentium* model of murine *E. coli* pathogenesis[63]. Regardless of these promising observations, the implications of pathogen-mediated alterations in normal cytokine profiles in providing sufficient trigger for IBS symptom establishment requires further investigation.

Mucosal barrier alterations

The intestinal epithelium provides an interface between the luminal space and the dynamic environment of the underlying subepithelial compartment. This physical barrier is intricately involved in regulating the controlled passage of vital nutrients, molecules, and water, *via* a semipermeable function maintained by TJs. TJs actively maintain the polarized characteristic of the epithelial barrier, and are composed of over 40 proteins consisting of occludin, junctional adhesion molecule (JAM), and claudins[64]. Patients with a history of infectious events experiencing Pi-IBS show drastic increases in permeability[65,66]. A prospective study, however, following a large waterborne outbreak of bacterial gastroenteritis, incited by mixed infection of EHEC O157:H7 and *C. jejuni*, documented increased permeability to be associated with IBS, regardless of whether symptoms were post-infectiously initiated[65]. Enterohemorrhagic *E. coli* (EHEC) is known to have deleterious impacts on the epithelial barrier through number of mechanisms, including TJ disruptions, and abnormal rates of intestinal epithelial cell (IEC) apoptosis[67,68]. These effects can be mediated directly *via* physical interaction through EHEC formation of characteristic attaching and effacing lesions (A/E lesions), and/or diffusely through toxin release[64,69]. EHEC, and its close relative: Enteropathogenic *E. coli* (EPEC), are known to hijack various pathways regulating the semi-permeable profile of TJs, and both have been shown to activate myosin light chain kinase (MLCK) to produce abnormally leaky barrier functionalities[70-72] (Figure 1). Additionally, *Giardia duodenalis*, a protozoan pathogen recently implicated in promoting Pi-IBS development[18,73], is well-known to disturb homeostatic barrier function through alterations in key TJ elements[74]. Specifically, *Giardia* has been shown to disrupt zonula occludins protein (ZO)-1, numerous transmembrane claudin proteins, and alter F-actin and α-actinin in order to disrupt paracellular flow[75,76] (Figure 1), which may have important implications in providing a mechanistic link between initial giardiasis, and subsequent development of IBS symptoms. Indeed, recent analysis of colonic biopsies from IBS patients indicated decreased expression of ZO-1, which was associated with increased permeability[77]. Moreover, an earlier report examining fecal extracts indicated higher levels of serine proteases in samples from IBS-D patients. When these extracts were applied to healthy colonic mucosa, they could elicit a proteinase activated receptor (PAR)-2 dependent increase in paracellular permeability in mice *via* increased myosin light chain (MLC) phosphorylation and delayed redistribution of ZO-1[78]. Numerous pathogens, including both EPEC and EHEC, produce potentially cytotoxic serine proteases[79], suggesting another possible link between enteric infection and IBS pathogenesis. Proteases are known to be involved in the infectious processes of pathogens such as EHEC and EPEC where they can prove detrimental to the epithelial barrier *via* modifications of the extracellular matrix[80], and or by activating protease-activated receptors, which have been shown to stimulate sensory neurons to produce hypersensitivity reactions[81]. Consequently, the possibility of residual pathogen mediators, such as inherent proteases, contributing to persistent changes in GI function requires further examination.

Enterochromaffin cells: Enterochromaffin cells (ECs) lining the GI mucosa are primary sources of Serotonin (5-HT) within the body. Alterations in the biosynthesis of 5-HT, in its release from ECs and degradation, and/or in its re-uptake, may have severe ramifications and perturb normal GI function[82]. Multiple studies have shown significantly higher 5-HT levels in the plasma of Pi-IBS patients compared with that of healthy controls, even in comparison to patients of the sporadic IBS-C subtype[83]. Recent studies have observed such significant alterations in EC counts and 5-HT levels, that the authors declared Pi-IBS as a distinct IBS subtype[10,11]. Augmented numbers of 5-HT-contatining ECs have been observed in colonic biopsies from patients following *C. jejuni* infection[9]. Up to 25% of *C. jejuni* infections are known to result in IBS[9], and the resulting implications on EC hyperplasia and excessive 5-HT bioavailability suggest a possible

mechanism whereby enteric infection may provide sufficient trigger for IBS symptom generation. Additionally, numerous reports have suggested a defect in the serotonin reuptake transporter (SERT) expression, and function in IBS patients[82,84,85], which may dictate inadequacies in homeostatic serotonin turnover. Interestingly, in the *T. spiralis* model of Pi-IBS, mice develop chronic abnormal motility patterns subsequent to infection, an effect that is accompanied by EC hyperplasia and 5-HT release[6,13], and blocked upon administration of a 5-HT antagonist[86]. In contrast, patients with persisting abdominal symptoms after acute *Giardia* infection have lower duodenal 5-HT-containing ECs, and lower plasma 5-HT postprandially, compared to controls[87], further underscoring the complexity of IBS pathophysiology.

Intestinal microbiota disruptions: The intestinal microbiota have extensive protective capacities[88] that are maintained by a diverse species profile. The characteristic high fat, high protein diets employed by the majority of people living in westernized countries facilitates the establishment of distinct microbiota species profile, as compared to that of those living in rural areas of developing countries, with a polysaccharide-rich diet[89]. Particular bacterial groups, mainly *Bacteroidetes* are known to harbor significant genetic capabilities to hydrolyse xyloses, making it an important constituent of the microbiota of people subsisting on carbohydrate-dominant food sources. The relative sensitivity of these distinct microbiota to enteropathogens, and how in turn disruptions in their respective flora may differentially regulate post-infectious disorders, is unknown.

Interestingly, changes in the relative Firmicutes to Bacteroidetes ratio[90,91], loss of Bifidobacteria spp. and Faecalibacterium[91], and overall diminished diversity[92], are all apparent in the microbiota profile of IBS patients. Additionally, numerous studies have demonstrated small intestinal bacterial overgrowth in IBS patients, where excessive colonization of the small intestine occurs with colonic flora[33,93]. There is the possibility that enteropathogens may disrupt the indigenous microbiota, either directly through pathogen-microbiota interactions, indirectly *via* the host mucosal immune response to the pathogen, or by a combination of the two[94]. For example, *S. enterica* serovar Typhimurium induced the loss of 95% of total bacterial numbers throughout the murine intestinal tract, 7 d following infection[94]. Findings from ongoing research also indicate that G. duodenalis and C. jejuni are able to directly alter species distribution of human commensal microbiota[95]. Pathogenic effects, however, may only provide a suitable trigger, and ultimately require the accompaniment of a host inflammatory response in order to markedly alter the microbiota ecosystem. The necessity of these compounding factors is exemplified in contrasting *C. rodentium* and *C. jejuni* murine infection models, where the former induces overt host inflammation, while the later can successfully colonize without producing inflammatory reactions[96]. It appears then, that both enteropathogen assault, combined with pathogen-mediated intestinal inflammation, can elicit dramatic changes in the total abundance of the intestinal microbiota, and shift in anaerobic:aerobic species[96].

CONSIDERATION

Many studies that classify patients as experiencing Pi-IBS do so based upon questionnaires, highlighting the fact that they rely exclusively on a patient's recall of past medical events, including infections and/or prescription drug use. Some antibiotics, for example, have established causality in disturbing the overall fecal microbial composition through drastic reduction of *Firmicutes* and *Bacteroidetes*, and a corresponding promotion of *Proteobacteria* spp.[97].

Also, the classification of IBS as biopsychosocial disorder challenges the mantra of body and mind being distinct entities, and suggests an equal consideration of both when examining disease manifestation. The risk of developing IBS symptoms following enteric infection may also differ in individuals depending on psychological parameters such as stress level, emotional status, and upbringing. High stress and anxiety levels, for instance, are associated with IBS development following *Campylobacter* infection[98]. Anxiety, as well as depression, is also correlated with altered pain perception in IBS patients[30]. Additionally, anxiety and depressive states in IBS patients were recently shown to lead to changes in serum levels of gastrointestinal hormones. Indeed, the authors suggest increased secretion of somatostatin and vasoactive intestinal peptide seen in IBS patients exhibiting anxiety-depression emotional state ratings, may contribute to altered gastrointestinal motility and function[99]. An important mediator in the endocrine arm of the stress response, corticotropin-releasing factor, may also contribute to Pi-IBS development through direct local action on specific cellular targets, namely mast cells, and consequently lead to the modification the intestinal inflammatory process[100].

Additionally, as Pi-IBS is defined based upon the development of exclusively new IBS symptom presentation, researchers must be certain that no preceding presentation of IBS occurred. Indeed, clear cause-to effect relationship studies need to establish mechanistic causalities in Pi-IBS.

CONCLUSION

Unfortunately, the link between physiological consequences of enteric infection and altered gut function (sensitivity and motility) seen in IBS remains largely circumstantial. As many as 30%-40% of patients experiencing enteritis can go on to develop chronic GI abnormalities compatible with IBS; however, this means that a greater percentage of patients make a full recovery. Susceptibility, in turn, to developing IBS is determined by a number of factors, with enteric pathogens constituting only one possible route of initiation. Regardless of the

heterogeneous initiation mechanisms culminating into disease, the pathophysiological implications of enteric infection provide important clues towards elucidating the mechanics underlying IBS manifestation. Animal models are becoming increasingly appreciated as divergent means in which IBS triggering mechanisms may be elucidated. Indeed, the maternal separation stress model in rodents is well documented in mimicking early life stress that can result in lifelong dysfunctions in the brain-gut axis, and is implicated in predisposing to IBS development[101]. Furthermore, animal models of post-infectious, or post-inflammatory conditions, such as those using *T. spiralis* or TNBS, are proving useful in examining the mechanisms underlying motility and pain perception changes subsequent to diverse stimuli, without the challenges associated with patient recall, or the need for complex psychological status analyses.

This is especially relevant in terms of developing treatment technologies to combat IBS, most of which currently target overt symptomology. Many of the physiological consequences of GI infections represent parallels with fundamental triggering mechanisms currently though to contribute to IBS. Understanding the similarities between remnants of enteric infections, and the detrimental outcomes, can lead to the development of prevention strategies and therapeutic techniques to target IBS generation; before it can even start.

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ORIGINAL ARTICLE

HLA variants related to primary sclerosing cholangitis influence rejection after liver transplantation

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Abstract

AIM: To investigate influence of human leukocyte antigen (HLA) and killer immunoglobuline-like receptor (KIR) genotypes on risks of acute rejection (AR) after liver transplantation (LTX).

METHODS: In this retrospective study we included 143 adult donor-recipient pairs with a minimum of 6 mo follow-up after LTX for whom DNA was available from both donor and recipients. Clinical data, all early complications including episodes and severity of AR and graft/patient survival were registered. The diagnosis of AR was based on clinical, biochemical and histological criteria. All suspected episodes of AR were biopsy confirmed. Key classical HLA loci (*HLA-A*, *HLA-B*, *HLA-C* and *HLA-DRB1*) were genotyped using Sanger sequencing. 16 KIR genes were genotyped using a novel real time PCR approach which allows for determination of the diploid copy number of each KIR gene. Immunohistochemical staining for T (CD3), B (CD20) and natural killer (NK) cells (CD56 and CD57) were performed on liver biopsies from 3 different patient groups [primary sclerosing cholangitis (PSC), primary biliary cirrhosis and non-autoimmune liver disease], 10 in each group, with similar grade of AR.

RESULTS: Fourty-four (31%) patients were transplanted on the basis of PSC, 40% of them had AR *vs* 24% in the non-PSC group (P = 0.04). No significant impact of donor-recipient matching for HLA and KIR genotypes was detected. In the overall recipient population an increased risk of AR was detected for HLA-B*08 (P = 0.002, OR = 2.5; 95%CI: 1.4-4.6), HLA-C*07 (P = 0.001, OR = 2.4; 95%CI: 1.4-4.0) and HLA-DRB1*03 (P = 0.03, OR = 1.9; 95%CI: 1.0-3.3) and a decreased

risk for HLA-DRB1*04 (P = 0.001, OR = 0.2; 95%CI: 0.1-0.5). For HLA-B*08, HLA-C*07 and DRB1*04 the associations remained evident in a subgroup analysis of non-PSC recipients (P = 0.04, P = 0.003 and P = 0.02, respectively). In PSC recipients corresponding P values were 0.002, 0.17 and 0.01 for HLA-B*08, HLA-C*07 and DRB1*04, respectively. A dosage effect of AR prevalence according to the PSC associated HLA alleles was also notable in the total recipient population. For HLA-B*08 the frequency of AR was 56% in HLA-B*08 homozygous recipients, 39% in heterozygous recipients and 21% in recipients lacking HLA-B*08 (P = 0.02). The same was observed for the HLA-C*07 allele with AR in 57%, 27% and 18% in recipients being homozygous, heterozygous and lacking HLA-C*07 respectively (P = 0.003). Immunohistochemical analysis showed similar infiltration of T, B and NK cells in biopsies with AR in all three groups.

CONCLUSION: We found significant associations between the PSC-associated HLA-B*08, HLA-C*07, HLA-DRB1*03 and HLA-DRB1*04 alleles and risk of AR in liver transplant recipients.

Key words: Liver transplantation; Primary sclerosing cholangitis; Acute rejection; Human leukocyte antigen; Killer immunoglobulin-like receptor

Core tip: Patients undergoing liver transplantation on the basis of primary sclerosing cholangitis (PSC) have a higher frequency of acute cellular rejections than non-PSC patients. Recent studies have determined the genetic susceptibility to PSC, of which genetic variants in the human leukocyte antigen complex represent the strongest risk factors. In the present report we show that these variants also influence risk of acute cellular rejection after liver transplantation in PSC. Moreover, we show that the same variants also involve in risk of acute cellular rejection in non-PSC recipients.

Fosby B, Næss S, Hov JR, Traherne J, Boberg KM, Trowsdale J, Foss A, Line PD, Franke A, Melum E, Scott H, Karlsen TH. HLA variants related to primary sclerosing cholangitis influence rejection after liver transplantation. *World J Gastroenterol* 2014; 20(14): 3986-4000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/3986.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.3986

INTRODUCTION

Liver transplantation (LTX) is the only curative treatment for end-stage liver disease[1]. Although both graft and patient survival have improved dramatically over the last two decades, acute rejection (AR) still represents a significant clinical problem[2-5]. Numerous studies over the years have shown different frequencies (range 11%-80%) of AR after LTX, but consistently the incidence of AR in patients transplanted on the basis of primary sclerosing cholangitis (PSC) is increased (range 17%-100%) compared to other groups[3-6]. Patients treated for non-autoimmune liver diseases (*e.g.*, alcoholic liver disease or fulminant hepatic failure from paracetamol intoxication) seem to have a low risk of AR[2,6-8]. Analyses of variables affecting AR risk after LTX have been inconsistent, and whether matching of donor and recipient for human leukocyte antigen (HLA) influences AR occurrence after LTX remains controversial[9-12]. The increased risk of AR in PSC patients after LTX is clinically important because increased immunosuppression is needed with concomitant side effects. Rejection episodes in PSC patients may also increase the risk for recurrent PSC in the liver allograft[5,13-15].

HLA class I molecules interact specifically with T-cell receptors (TCR) upon binding of antigenic peptides, and they (HLA-B and HLA-C in particular) can also act as ligands for killer-cell immunoglobulin-like receptors (KIR) expressed on natural killer (NK) cells and subsets of T-cells[16-18]. The interaction between the genetically determined HLA-C2 variant and its corresponding KIRs delivers a more potent inhibitory signal to NK cells than HLA-C1[16]. The potential benefit of incorporating genetic killer immunoglobulin-like (KIR) variation when assessing the impact of HLA matching on AR risk is not clear[19-23]. The major biological role of the HLA-KIR interaction is to influence activation status in various contexts of NK cell and T cell function (*e.g.*, cytotoxicity)[16]. The HLA-C/KIR interactions have been studied extensively in HLA-related[24,25] and unrelated[26,27] hematopoietic stem cell transplantation (HSCT).

There is evidence to suggest that the influence of HLA on AR risk is not uniform in all recipients and that stratification for the presence of underlying autoimmune liver diseases with known HLA associations may influence results[12,28]. PSC is strongly HLA-associated[29], with the peak association signal mapping to the HLA class I region (HLA-B*08 in particular), but there are also significant residual effects within class Ⅱ, likely represented by several HLA-DRB1 alleles (*i.e.*, DRB1*13:01, DRB1*03:01, DRB1*04, DRB1*07 and DRB1*11)[30-33], or variants in linkage disequilibrium with these. Two studies also suggest that genetic variation affecting the binding of class I molecules to KIRs contribute to the HLA associations in PSC[34,35].

In this study we aimed to explore the influence of HLA and KIR genotypes on AR risk after LTX with particular emphasis on patients transplanted for PSC compared to recipients with other underlying liver diseases.

MATERIALS AND METHODS

Patients

All patients undergoing LTX between 1996 and 2008 at Oslo University Hospital, Rikshospitalet, Norway were included in a retrospective design. A total of 520 LTX

were performed during this period and for 198 donor/recipient pairs archival DNA samples were available for HLA and KIR genotyping. We included all patients above 12 years that received a first liver graft from a deceased donor and with a minimum follow-up period of six months (n = 176). Patients with early graft loss (< 30 d) resulting from primary dysfunction or vascular complications without evidence of AR were excluded (n = 7). Patients receiving ABO incompatible liver or those with a combined liver/kidney transplantation were also excluded (n = 4). To minimize the impact of confounders, we excluded patients with uncertain or inconclusive biopsy results and patients treated for AR based on clinical/biochemical suspicion only, without a confirmatory biopsy (n = 17; 8 PSC, 3 autoimmune hepatitis, 1 hepatitis C cirrhosis, 2 hepatitis C cirrhosis, 2 alcoholic cirrhosis, 1 primary biliary cirrhosis). Finally we also excluded 5 patients who received immunosuppressive regimens according to obsolete protocols (calcineurin inhibitor in combination with azathioprine and prednisolone).

The final study population comprised 143 adult recipients (82 males, 61 females) with a median age of 52 years (range 13 to 73 years). PSC patients constituted the largest group of liver recipients (n = 45). The diagnosis of PSC was in all cases made based on standard diagnostic criteria[36]. The study protocol received prior approval by the regional ethics committee (reference number S-08873b). Written informed consent concerning the use of biopsy/DNA material was obtained from all recipients still alive. The research ethics committee granted exemption from consent for recipients deceased at time of study initiation. Furthermore, the Norwegian Health Directorate approved the utilization of DNA samples from the deceased donors (reference number 08/10827).

Clinical data

By thorough investigation of all patient records, blood tests, radiology results and histology assessments, we determined indications for LTX, number and severity of episodes of AR [Banff rejection activity index (RAI) scoring, steroid responsive/non-responsive], graft survival and overall patient survival. Immunosuppression consisted of standard triple-drug therapy with tacrolimus, prednisolone and mycophenolate mofetil. Tacrolimus (or cyclosporin) was given on the first day after LTX and adjusted according to daily blood concentrations. Methylprednisolone was administered perioperatively at a dosage of 500 mg, subsequently tapered to 20 mg/d during the first week and gradually to 5 mg/d within 6 mo. Mycophenolate was given at an initial dose of 1000 mg twice daily.

The diagnosis of AR was based on clinical, biochemical and histological criteria. According to routine practice at our center, all patients were monitored by daily blood samples and any increase in liver enzymes or bilirubin resulted in examination with liver sonography with doppler to determine the presence of circulatory or anatomical complications. After clinical exclusion of circulatory and surgical causes of detected abnormalities in liver enzymes and/or bilirubin, a liver biopsy was carried out. All suspected episodes of AR included in this study were biopsy confirmed and classified by rejection activity index (RAI) according to the Banff 1997 standard[37]. All but two biopsies, which showed severe AR, were classified as either mild (RAI 3-4) or moderate (RAI 5-6). Ten patients (5 PSC, 2 primary biliary cirrhosis (PBC), 1 hepatocellular carcinoma, 1 cholangiocarcinoma and 1 hepatitis C infection) had steroid-resistant AR (after 3-4 pulses of methylprednisolone over 3-4 consecutive days, total dose of 2-2.5 g) and were subsequently treated with antithymocyte globuline (ATG) according to protocol.

HLA-genotyping

HLA-A, *-B*, *-C* (class Ⅰ) and *-DRB1* (class Ⅱ) typing was performed using previously described sequencing-based typing protocols at Institute for Immunology, Oslo University Hospital, Rikshospitalet, Norway[34]. Ambiguous HLA types were excluded from analysis (1 patient HLA-B, 1 patient *HLA-C*).

KIR-genotyping

KIR genotyping was performed using a novel method allowing accurate determination of copy number variation (CNV) of each *KIR* gene based on a triplex real-time PCR approach[38]. 14 KIR-genes and 2 pseudogenes were genotyped for each sample in quadruplicate; samples with ⩽ 1 successful replicate were excluded. Calling of copy numbers was verified manually in CopyCaller v.1.0 (Applied Biosystems, Foster City, CA, United States) and results imported into Microsoft Excel (Redmond, WA, United States). Copy numbers > 4 were excluded and all 0 copies were verified by amplification of the reference gene.

Immunohistochemistry

To expand on the genetic analysis of KIR genes, also considering the conflicting results in previously published experiments[19-23], an immunohistochemical determination of the number of NK cells as compared with T- and B cells in liver allograft biopsies with AR were performed. Archival biopsy material from 10 patients with PSC, 10 patients with PBC and 10 patients with non-immunological liver diseases (3 alcoholic cirrhosis, 1 polycystic liver disease, 2 cryptogenic cirrhosis, 1 Budd Chiari and 3 with colorectal liver metastases) were examined. All selected biopsies had similar severity of rejection characterized according to the Banff criteria[37], with a median RAI score of 5, 4.5 and 5 for PSC, PBC and the non-immunological group, respectively.

Immunohistochemical staining procedure: Antigen retrieval: heat treatment in Tris EDTA, pH9.0 buffer. Primary antibodies: Monoclonal anti CD20, dilution 1:200 and anti CD56, dilution 1:100, both from Daco, Glostrup, Denmark; monoclonal anti CD57, dilution 1/50, Nova Castra, Newcastle, United Kingdom; polyclonal

Table 1 Distribution and frequency of the underlying liver disease of 143 liver transplant recipients

Indication for transplantation	*n* (%)
Autoimmune etiology	65 (45.5)
Primary sclerosing cholangitis	44 (30.8)
Primary biliary cirrhosis	14 (9.8)
Autoimmune hepatitis	7 (4.9)
Viral hepatitis	20 (13.9)
Hepatitis B	5 (3.5)
Hepatitis C	15 (10.5)
Other indications	65 (43.9)
Hepatocellular carcinoma	13 (8.8)
Secondary liver tumours	7 (4.7)
Acute hepatic failure	8 (5.4)
Alcoholic cirrhosis	17 (12.2)
Cholangiocarcinoma	10 (6.8)
Budd-Chiari	2 (1.4)
Cryptogenic cirrhosis	9 (6.1)
Metabolic liver disease	1 (0.5)
Polycystic liver disease	2 (1.4)

rabbit anti CD3, dilution 1/150, Neo Markers, Fremont CA, United States. Diluent: Ventana Antibody Diluent, Ventana Medical System, Tucson AZ. Incubation time: 30 min at 37 C. Detection system: Ultra View Universal DAB- or Alkaline Phosphatase Red-Detection Kits. Double stainings: Sequential stainings, one primary antibodies followed by one of the detection kits, a second primary antibody and the other detection kit. Since NK cell specific monoclonal antibodies could not be used in formalin fixed material, we stained the biopsy samples for CD56 and CD57 for detection of putative NK- and NKT cells.

We calculated the B cell percentage (CD20/CD20 + CD3) in the portal infiltrates based on counting of more than 400 B and T cells in the portal areas. Since the numbers of CD56 and CD57 positive cells turned out to be very low, we decided to use the absolute number per square millimeter. Double immunostaining of CD56 and CD3 was performed in order to distinguish between classical NK cells ($CD3^-CD56^+$) and natural killer T (NKT) cells ($CD3^+CD56^+$), which represent a distinct lineage of T cells and share several surface markers with NK cells.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL). *P* values less than 0.05 were considered significant. The prevalence of AR in the different groups was compared using the χ^2 test and, where appropriate, the Fisher's exact test. OR and corresponding 95%CI were calculated using Woolf's formula with Haldane's correction. As have been demonstrated in previous studies[29,43], there is no significant population stratification in the Norwegian population. Comparison of means for KIR gene copy number correlated to AR was performed using independent sample *t* test (after assessment of normal distribution). For analysis of NK cell infiltration in biopsies with AR, the one way ANOVA test was used. All *P* values from the KIR association analysis were corrected for multiple testing according to Bonferroni ($n = 68$).

RESULTS

The disease indications for LTX are shown in Table 1. The overall frequency of histologically confirmed AR in the study population was 28%, with significantly higher frequency noted in patients with PSC as compared with patients transplanted on the basis of other liver diseases (40% *vs* 24%, OR = 2.2, 95%CI: 1.0-4.6, $P = 0.04$). All episodes of AR occurred within the first 6 weeks after LTX, with a median of 10 days (range 5-40 d). PSC patients with concurrent IBD (82%), showed tendency to an increased risk of AR compared to PSC patients without IBD (43% *vs* 25%), but this was not statistically significant ($P = 0.34$). We found no statistically significant influence from age or gender on risk of acute rejection (data not shown). For further analyses, recipients were grouped according to the occurrence of AR or non-AR and subgrouped according to underlying PSC or not.

PSC-associated HLA alleles confer risk for AR independently of underlying disease

Given the significantly higher frequency of AR in patients with PSC, we specifically analyzed the total study population for differences in frequencies of PSC associated *HLA-A*, *-B*, *-C* and *-DR* alleles between patients with AR and without AR. From the HLA alleles previously reported to associate with increased PSC susceptibility, an increased risk of AR was detected for recipients positive for HLA-B*08 ($P = 0.002$, OR = 2.5, 95%CI: 1.4-4.6), HLA-C*07 ($P = 0.001$; OR = 2.4; 95%CI: 1.4-4.0) and HLA-DRB1*03 ($P = 0.03$, OR = 1.9, 95%CI: 1.0-3.3) (Tables 2-4). For the HLA-DRB1*04 allele, previously shown to associate with decreased risk of PSC, reduced risk of AR in the overall study population was found ($P = 0.001$, OR = 0.2, 95%CI: 0.1-0.5) (Table 4). Notably, for HLA-B*08, HLA-C*07 and HLA-DRB1*04 these associations remained evident in a subgroup analysis of non-PSC recipients only ($n = 99$) ($P = 0.04$, $P = 0.003$ and $P = 0.02$, respectively) (Tables 5-7). In PSC recipients ($n = 44$) corresponding *P* values were 0.002, 0.17 and 0.03 for HLA-B*08, HLA-C*07 and DRB1*04, respectively.

A dosage effect of AR prevalence according to the PSC-associated HLA alleles was also notable in the total recipient population. For HLA-B*08, the frequency of AR was 56% in HLA-B*08 homozygous recipients, 39% in heterozygous recipients and 21% in recipients lacking HLA-B*08 ($P = 0.02$). The same was observed for the HLA-C*07 allele with AR in 57%, 27% and 18% of recipients being homozygous, heterozygous and lacking HLA-C*07 respectively ($P = 0.003$). For HLA-DRB1*04 the situation was reversed, with the frequency of AR being 0% for DRB1*04 homozygous recipients, 12% for heterozygous and 35% in recipients lacking the DRB1*04 allele ($P = 0.009$).

Table 2 Frequencies of human leukocyte antigen-B alleles in the acute cellular rejection group (*n* = 40) and the non-acute cellular rejection group (*n* = 102) in the total population (missing *n* = 1)

HLA-B allele	Acute rejection		No acute rejection		OR	95%CI	Uncorrected
	n (alleles)	(%)	*n* (alleles)	(%)			*P* value
*05	0	(0)	8	(4)	0.1	0.0-1.1	0.070
*07	10	(13)	23	(11)	1.2	0.6-2.5	0.690
*08	27	(34)	34	(17)	2.5	1.4-4.6	0.002
*12	12	(14)	27	(13)	1.2	0.6-2.3	0.700
*13	1	(1)	5	(2)	1.1	0.3-4.6	1.000
*14	2	(2)	4	(2)	1.1	0.3-4.6	1.000
*15	6	(7)	28	(14)	0.5	0.2-1.2	0.150
*16	1	(1)	4	(2)	0.7	0.1-3.4	0.510
*17	1	(1)	6	(3)	0.6	0.1-2.8	0.410
*18	1	(1)	6	(3)	0.4	0.1-2.0	0.240
*21	0	(0)	2	(1)	0.5	0.1-4.8	0.360
*22	3	(3)	2	(1)	3.6	0.8-16.4	0.120
*27	4	(5)	10	(5)	1.1	0.4-3.2	1.000
*35	4	(6)	19	(9)	0.7	0.3-1.8	0.230
*37	4	(5)	4	(2)	2.6	0.7-9.1	0.180
*40	4	(5)	19	(9)	0.6	0.2-1.5	0.220
*41	1	(1)	2	(1)	1.5	0.3-9.1	0.870
*47	0	(0)	1	(0.5)	0.8	0.1-9.2	0.530

OR and corresponding 95%CI were calculated using Woolf's formula with Haldane's correction; uncorrected *P* value: calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen; AR: Acute cellular rejection.

Table 3 Frequencies of human leukocyte antigen-C alleles in the acute cellular rejection group (*n* = 40) and the non-acute cellular rejection group (*n* = 102) in the total population (missing *n* = 1)

HLA-C allele	Acute rejection		No acute rejection		OR	95%CI	Uncorrected
	n (alleles)	(%)	*n* (alleles)	(%)			*P* value
*01	2	(2.5)	8	(3.9)	0.6	0.3-2.9	0.43
*02	2	(2.5)	11	(5.4)	0.5	0.1-1.8	0.24
*03	10	(12.5)	43	(21.1)	0.7	0.3-1.1	0.09
*04	8	(10.0)	24	(11.8)	1.1	0.4-2.2	0.89
*05	8	(10.0)	18	(8.8)	1.0	0.5-2.3	0.76
*06	6	(7.5)	16	(7.8)	1.2	0.5-2.8	0.82
*07	41	(51.2)	62	(30.4)	2.4	1.4-4.0	0.001
*08	2	(2.5)	4	(2.0)	1.1	0.3-4.7	0.54
*12	0	(0)	8	(4.2)	0.2	0.02-1.1	0.07
*14	0	(0)	1	(0.5)	0.9	0.08-9.4	0.72
*15	0	(0)	4	(2.0)	0.3	0.03-2.5	0.26
*16	0	(0)	3	(1.5)	0.4	0.04-3.2	0.31
*17	1	(1.2)	4	(2.0)	0.8	0.2-4.3	0.57

OR and corresponding 95%CI were calculated using Woolf's formula with Haldane's correction; uncorrected *P* value: calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen.

For all other recipient class I and class II alleles, no significant differences between the groups with and without AR were detected.

Effect of HLA class I and class II mismatching on the risk of AR

HLA-class I or class II recipient-donor mismatching (0 *vs* 1-2 mismatches for *HLA-A*, *HLA-B*, *HLA-DR* and up to 4 *vs* 5-6 mismatches for all three epitopes combined, respectively) had no significant effects on AR incidence (Table 8), neither when all patient-donor pairs were analyzed together nor when subgrouped according to PSC/non-PSC status (Table 9).

KIR ligand (i.e., HLA-C and Bw4) disparity does not affect the risk of AR after LTX

According to the established differences in KIR binding strength for HLA-C1 and HLA-C2[21,39,40], genotyped HLA-C alleles were classified as either HLA-C1 (Asn^{80}) or HLA-C2 (Lys^{80}). The donor HLA-C ligand group (*i.e.*, C1 or C2) did not significantly influence the risk of AR after LTX. AR was observed in 44% of LTX with a HLA-C2 homozygote donor and in 22% in the heterozygous group compared to 31% in the HLA-C1 homo-

Table 4 Frequencies of human leukocyte antigen-DRB1 alleles in the acute cellular rejection group (n = 40) and the non-AR group (n = 103) in the total population

HLA-DRB1	Acute rejection		No acute rejection		OR	95%CI	Uncorrected
allele	n (alleles)	(%)	n (alleles)	(%)			P value
*01	10	(12.5)	23	(11.2)	1.2	0.5-2.5	0.75
*02	15	(18.8)	31	(15.0)	1.3	0.7-2.6	0.44
*03	25	(31.3)	40	(19.4)	1.9	1.0-3.3	0.03
*04	4	(5.0)	46	(22.3)	0.2	0.1-0.5	0.001
*07	7	(8.8)	16	(7.8)	1.2	0.5-2.8	0.78
*08	2	(2.5)	12	(5.8)	0.5	0.1-1.8	0.20
*09	0	(0)	1	(0.5)	0.9	0.1-9.5	0.72
*10	1	(1.3)	4	(1.9)	0.8	0.2-4.4	0.57
*11	1	(1.3)	6	(2.9)	0.6	0.1-2.9	0.37
*12	1	(1.3)	3	(1.5)	1.1	0.2-6.1	0.69
*13	13	(16.3)	21	(10.2)	1.7	0.8-3.6	0.16
*14	1	(1.3)	3	(1.5)	1.1	0.2-6.1	0.69

OR and corresponding 95%CI were calculated using Woolf's formula with Haldane's correction; uncorrected P value is calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen.

Table 5 Comparison of the frequencies of human leukocyte antigen-B alleles in the acute cellular rejection group (n = 23) and the non-acute cellular rejection group (n = 76) in the non-primary sclerosing cholangitis population

HLA-B	Acute rejection		Non acute rejection		OR	95%CI	Uncorrected
allele	n (alleles)	(%)	n (alleles)	(%)			P value
*05	0	(0)	4	(2.6)	0.3	0.1-2.9	0.34
*07	6	(13.0)	15	(9.9)	1.4	0.5-3.1	0.54
*08	12	(26.0)	20	(13.2)	2.1	0.8-4.6	0.04
*12	9	(19.6)	20	(13.2)	1.6	0.6-3.4	0.36
*13	1	(2.2)	4	(2.6)	1.0	0.2-5.4	0.67
*14	2	(4.3)	4	(2.6)	1.8	0.5-5.81	0.43
*15	5	(10.9)	21	(13.8)	0.8	0.3-2.1	0.51
*16	1	(2.2)	3	(2.6)	1.0	0.2-5.34	1.00
*17	0	(0)	4	(2.6)	0.3	0.1-2.9	0.63
*18	1	(2.2)	6	(3.9)	0.6	0.1-2.0	1.00
*21	0	(0)	2	(1.3)	0.7	0.1-2.5	1.00
*22	0	(0)	2	(1.3)	0.7	0.11-2.5	0.71
*27	2	(4.3)	7	(4.6)	1.0	0.3-4.0	1.00
*35	4	(8.7)	15	(9.9)	0.8	0.3-2.5	1.00
*37	2	(4.3)	3	(1.9)	2.4	0.4-10.3	0.34
*40	2	(4.3)	17	(11.2)	0.4	0.1-1.4	0.25
*41	1	(2.2)	2	(1.3)	1.9	0.3-11.3	0.56
*47	0	(0)	1	(0.7)	1.0	0.1-11.4	1.00

OR and corresponding 95%CIs were calculated using Woolf's formula with Haldane's correction. Uncorrected P value is calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen.

zygous group (P = 0.15). For analysis of HLA-C KIR ligand disparity between recipient and donor, we assessed the following possibilities; (1) HLA-C ligand type present in the recipient and absent in the donor (*i.e.*, "missing self" model); (2) ligand present in the donor and absent in the recipient (*i.e.*, "non-self" model); and (3) donor and recipients homozygous for HLA-C1 and HLA-C2, respectively (*i.e.*, complete disparity). We did not detect any significant influence from these types of HLA-C/KIR ligand disparity on risk of AR, either in the total cohort nor in an analysis stratified according to PSC (P > 0.05 in all cases).

Classification of the *HLA-B* alleles was done according to whether they determined the Bw4 (that binds to KIR) or Bw6 (that does not bind KIR). We found no influence of donor HLA-Bw genotype on AR risk, *i.e.*, AR occurred in 29% of LTX with a HLA-Bw4 homozygote donor, in 28% in the heterozygote group and in 35% in the HLA-Bw6 homozygote group (P = 0.15). Ligand disparity was defined as recipient carriage of KIR3DL1 and Bw4 (*i.e.*, presence of inhibition *via* KIR3DL1-Bw4 interaction), with Bw4 absent in the donor (*i.e.*, no inhibition *via* KIR3DL1-Bw4 interaction). There was no significant difference in the frequency of AR between disparate (25%) and non-disparate individuals (29%), P = 0.70.

Impact of KIR genes on risk of AR

No significant associations between KIR genotype of

Table 6 Comparison of the frequencies of human leukocyte antigen-C alleles in the acute cellular rejection group (n = 23) and the non-acute cellular rejection group (n = 76) in the non-primary sclerosing cholangitis population

HLA-C allele	Acute rejection		No acute rejection		OR	95%CI	Uncorrected
	n (alleles)	(%)	n (alleles)	(%)			P value
*01	1	(2.2)	5	(3.3)	0.9	0.16-4.27	1.00
*02	1	(2.2)	7	(4.6)	0.6	0.11-2.53	0.68
*03	4	(8.7)	34	(22.4)	0.4	0.13-0.93	0.06
*04	7	(15.2)	21	(13.8)	1.2	0.45-2.61	0.81
*05	6	(13.0)	12	(7.9)	1.8	0.55-3.74	0.38
*06	3	(6.5)	12	(7.9)	0.9	0.27-2.74	1.00
*07	23	(50.0)	40	(26.3)	2.8	1.42-5.42	0.003
*08	2	(4.3)	4	(2.6)	1.9	0.34-5.89	0.63
*12	0	(0)	6	(3.9)	0.2	0.02-1.62	0.34
*15	0	(0)	4	(2.6)	0.4	0.04-2.94	0.58
*16	0	(0)	3	(2.0)	0.5	0.05-3.97	0.32
*17	1	(2.2)	4	(2.6)	1.1	0.19-5.46	1.00

OR and corresponding 95%CI were calculated using Woolf's formula with Haldane's correction. Uncorrected P value calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen.

Table 7 Comparison of the frequencies of human leukocyte antigen-DRB1 alleles in the acute cellular rejection group (n = 23) and the non-acute cellular rejection group (n = 76) in the non-primary sclerosing cholangitis population

HLA-DRB1 allele	Acute rejection		No acute rejection		OR	95%CI	Uncorrected
	n (alleles)	(%)	n (alleles)	(%)			P value
*01	7	(15.2)	18	(11.8)	1.4	0.6-3.4	0.55
*02	8	(17.4)	23	(15.1)	1.2	0.5-2.8	0.46
*03	13	(28.2)	24	(15.8)	2.5	1.9-5.4	0.05
*04	3	(6.5)	35	(23.0)	0.3	0.1-0.9	0.02
*07	6	(13.0)	12	(7.9)	1.8	0.7-4.8	0.29
*08	2	(4.3)	10	(6.6)	0.7	0.2-2.7	0.73
*10	0	(0)	3	(2.0)	0.5	0.1-4.2	0.59
*11	1	(2.2)	6	(3.9)	0.7	0.1-3.7	1.00
*12	1	(2.2)	3	(2.0)	1.4	0.2-7.9	0.93
*13	7	(15.2)	15	(9.9)	1.7	0.7-4.2	0.31
*14	0	(0)	3	(2.0)	0.5	0.1-4.2	1.00

OR and corresponding 95%CIs were calculated using Woolf's formula with Haldane's correction. Uncorrected P value is calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen.

Table 8 Data on human leukocyte antigen mismatches on the risk of acute cellular rejection after liver transplantation n (%)

Locus	MM (n)	Non-AR group (n = 102)	AR group (n = 41)	P value
HLA-A	1-2	86 (84.3)	38 (92.7)	0.18
	0	16 (15.7)	3 (7.3)	
HLA-B	1-2	97 (95.1)	41 (100)	0.15
	0	5 (4.9)	0 (0)	
HLA-DR	1-2	94 (92.2)	40 (97.6)	0.21
	0	8 (7.8)	1 (2.4)	
A, B, DR	0-4	62 (60.8)	19 (47.3)	0.12
	5-6	40 (39.2)	22 (53.7)	

Uncorrected P values were calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen; MM: Mismatches; AR: Acute cellular rejection.

patients and risk of AR were detected (Tables 2 and 10). There was a trend toward the 22 bp deletion variant of the *KIR2DS4* gene in recipients experiencing AR. This was the case in both the overall population and in the PSC-stratified analysis, but this finding was not significant after correction for the number of comparisons made in the KIR analysis (Bonferroni factor 68, see Tables 10-12). There was no significant influence of recipient copy number of each individual KIR gene and risk of AR (Table 12).

Immunohistochemical analysis of biopsies with AR according to indication for liver transplantation

Based on the previous suggestions that HLA *vs* KIR genotype may influence the risk of AR[22,23,41,42], we assessed the frequency of NK cells in the inflammatory infiltrates in AR in PSC and non-PSC patients. We used CD56 and CD57 as markers to detect the number and distribution of possible NK cells, along with CD3 and CD20 for the counting of T-and B cells in the formalin fixed biopsies. Both epithelial cells in small bile ducts and scattered spindle shaped cells and lymphocyte-like cells were CD56-

Table 9 Data on human leukocyte antigen mismatches on the risk of acute cellular rejection after liver transplantation, according to primary sclerosing cholangitis or non-primary sclerosing cholangitis *n* (%)

Locus	MM (*n*)	Non-AR group (PSC, *n* = 26) (Non-PSC, *n* = 76)	AR group (PSC) *n* = 17) (Non-PSC) *n* = 24)	*P* value
HLA-A				
(PSC)	1-2	20 (57.1)	15 (42.9)	0.35
	0	6 (75.0)	2 (25.0)	
(non-PSC)	1-2	65 (73.9)	23 (26.1)	0.18
	0	11 (92.7)	1 (7.3)	
HLA-B				
(PSC)	1-2	25 (59.5)	17 (40.5)	0.41
	0	1 (100.0)	0 (0.0)	
(non-PSC)	1-2	73 (75.3)	24 (24.7)	0.32
	0	3 (100.0)	0 (0.0)	
HLA-DR				
(PSC)	1-2	26 (61.9)	16 (38.1)	0.44
	0	0 (0.0)	1 (100.0)	
(non-PSC)	1-2	67 (74.4)	23 (25.6)	0.27
	0	9 (90.0)	1 (10.0)	
A, B, DR				
(PSC)	5-6	12 (54.5)	10 (45.5)	0.42
	0-4	14 (66.7)	7 (33.3)	
(non-PSC)	5-6	29 (67.4)	14 (32.6)	0.08
	0-4	47 (82.4)	10 (17.6)	

Uncorrected *P* values were calculated by the χ^2 test or the Fisher's exact test where appropriate. HLA: Human leukocyte antigen; PSC: Primary sclerosing cholangitis; MM: mismatches; AR: acute cellular rejection.

Table 10 Data on the relationship between killer immunoglobulin-like receptor gene phenotype (presence/absence of gene) in the recipient and the risk of acute cellular rejection after liver transplantation in the total patient population

Recipient *KIR* gene phenotype		Incidence of AR	*P* value
2DL1	Negative (*n* = 3)	0%	0.37
	Positive (*n* = 140)	29%	
2DL2	Negative (*n* = 79)	28%	0.92
	Positive (*n* = 64)	29%	
2DL3	Negative (*n* = 10)	20%	0.44
	Positive (*n* = 133)	28%	
2DL4	Negative (*n* = 0)	-	-
	Positive (*n* = 143)	29%	
2DL5	Negative (*n* = 79)	32%	0.27
	Positive (*n* = 64)	24%	
2DP1	Negative (*n* = 3)	0%	0.54
	Positive (*n* = 140)	31%	
2DS1	Negative (*n* = 91)	32%	0.24
	Positive (*n* = 52)	20%	
2DS2	Negative (*n* = 76)	27%	0.85
	Positive (*n* = 67)	29%	
2DS3	Negative (*n* = 108)	28%	0.79
	Positive (*n* = 35)	31%	
2DS4DEL[1]	Negative (*n* = 23)	9%	0.03
	Positive (*n* = 120)	31%	
2DS4WT[2]	Negative (*n* = 77)	27%	0.84
	Positive (*n* = 66)	29%	
2DS4TOTA[3]	Negative (*n* = 3)	0%	0.67
	Positive (*n* = 140)	29%	
2DS5	Negative (*n* = 104)	32%	0.47
	Positive (*n* = 39)	24%	
3DL1E4[4]	Negative (*n* = 3)	0%	0.61
	Positive (*n* = 140)	29%	
3DL1E9[5]	Negative (*n* = 3)	0%	0.62
	Positive (*n* = 140)	28%	
3DL2	Negative (*n* = 0)	-	-
	Positive (*n* = 143)	29%	
3DL3	Negative (*n* = 0)	-	-
	Positive (*n* = 143)	29%	
3DP1	Negative (*n* = 0)	-	-
	Positive (*n* = 143)	29%	
3DS1	Negative (*n* = 91)	31%	0.39
	Positive (*n* = 52)	25%	

[1]2DS4DEL refers to the 22-bp deletion variant of 2DS4; [2]2DS4WT refers to the full-length form of the gene; [3]2DS4TOT refers to the total number of 2DS4, *i.e.*, 2DS4DEL and 2DS4WT combined; [4,5]3DL1E4 and 3DL1E9 refers to exon 4 and 9 of the *3DL1* gene respectively. The various KIR genes genotyped are listed in the leftmost column. Genes with S in the name (*e.g.*, KIR2DS4) encode activating KIRs, genes with L in the name (*e.g.*, KIR3DL1) encode inhibiting KIRs and genes with a P in the name (*e.g.*, KIR3DP1) are pseudogenes. Uncorrected *P* value is calculated by the χ^2 test or the Fisher's exact test where appropriate. KIR: Killer immunoglobulin-like receptor; AR: Acute cellular rejection.

positive (Figure 1). Bile ducts were positive for CD56 in equal fractions in patients with PSC (30%), PBC (30%) and other liver diseases (30%). The numbers and type of lymphocytes observed in the AR biopsies obtained from patients with PSC, PBC and non-immunological liver diseases revealed no significant differences between the groups (Table 13). CD56 positive lymphocytes were only sporadically detected in the portal tracts. Scattered CD57-positive cells were seen in both the portal tracts, periportal and sinusoidal areas in a similar fraction of patients in all three groups, and there were no statistically significant differences between the numbers of CD57-positive cells per square millimeter of liver tissue. Double staining, CD3 with immunoperoxidase and diaminobenzidine, followed by CD57 with alkaline phosphatase, showed no CD57-positive cells, indicating that all or practically all CD57-positive cells in the liver biopsies were $CD3^+CD57^+$ NKT cells.

DISCUSSION

The potential importance of donor-recipient HLA matching in LTX is debated. At some centers, including our own, resources are still spent providing HLA data for liver allograft recipients even though the information is not accounted for in the organ allocation process. It has been consistently shown that recipients with PSC have an increased AR risk compared to recipients with other underlying liver diseases. PSC is strongly HLA-associated[34,43-45], and in the present analysis we demonstrate that this HLA background also associates with increased risk of AR in non-PSC recipients. These results provide an impetus to more thoroughly explore the role of recipient immunogenetic determinants on liver graft outcomes, rather than overly focusing on genetic matching between the donor and the recipient.

The strongest HLA risk factor in PSC is HLA-B*08[43,46]. This association is a representative of the so-called 8.1 ancestral haplotype (AH8.1), which is defined by a series

Table 11 Data on the relationship between killer immunoglobulin-like receptor gene phenotype in the recipient and the risk of acute cellular rejection after liver transplantation in patients with primary sclerosing cholangitis compared with patients without primary sclerosing cholangitis

Recipient *KIR* gene phenotype	Incidence of AR	*P* value
2DL1		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 4)	40%	
(other)		1.00
Negative (n = 3)	0%	
Positive (n = 96)	25%	
2DL2		
(PSC)		0.37
Negative (n = 23)	33%	
Positive (n =21)	48%	
(other)		0.71
Negative (n = 54)	26%	
Positive (n = 45)	22%	
2DL3		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		1.00
Negative (n = 10)	20%	
Positive (n = 89)	24%	
2DL4		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		-
Negative (n = 0)	-	
Positive (n = 99)	24%	
2DL5		
(PSC)		0.17
Negative (n = 28)	48%	
Positive (n = 16)	25%	
(other)		0.81
Negative (n = 51)	25%	
Positive (n = 48)	22%	
2DP1		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		1.00
Negative (n = 3)	0%	
Positive (n = 96)	25%	
2DS1		
(PSC)		0.14
Negative (n = 13)	26%	
Positive (n = 31)	20%	
(other)		0.51
Negative (n = 60)	26%	
Positive (n = 39)	20%	
2DS2		
(PSC)		0.37
Negative (n = 23)	33%	
Positive (n = 21)	48%	
(other)		0.48
Negative (n = 54)	26%	
Positive (n = 45)	20%	
2DS3		
(PSC)		0.78
Negative (n = 34)	41%	
Positive (n = 11)	36%	
(other)		0.57
Negative (n = 76)	22%	
Positive (n = 25)	28%	
2DS4DEL[1]		
(PSC)		0.11
Negative (n = 8)	38%	
Positive (n = 36)	43%	
(other)		0.10
Negative (n = 15)	7%	
Positive (n = 85)	27%	
2DS4WT[2]		
(PSC)		0.81
Negative (n = 21)	38%	
Positive (n = 23)	42%	
(other)		0.71
Negative (n = 56)	22%	
Positive (n = 43)	26%	
2DS4TOTA[3]		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		1.00
Negative (n = 3)	0%	
Positive (n = 96)	25%	
2DS5		
(PSC)		0.46
Negative (n = 34)	43%	
Positive (n = 10)	30%	
(other)		0.56
Negative (n = 69)	25%	
Positive (n = 30)	20%	
3DL1E4[4]		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		-
Negative (n = 0)	-	
Positive (n = 99)	25%	
3DL1E9[5]		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		1.00
Negative (n = 3)	0%	
Positive (n = 96)	25%	
3DL2		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		-
Negative (n = 0)	-	
Positive (n = 98)	24%	
3DL3		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		-
Negative (n = 0)	-	
Positive (n = 99)	24%	
3DP1		
(PSC)		-
Negative (n = 0)	-	
Positive (n = 44)	40%	
(other)		-
Negative (n = 0)	-	
Positive (n = 99)	24%	

3DS1		
(PSC)		0.42
Negative (n = 31)	44%	
Positive (n =13)	31%	
(other)		0.81
Negative (n = 61)	25%	
Positive (n = 40)	23%	

[1]2DS4DEL refers to the 22-bp deletion variant of 2DS4; [2]2DS4WT refers to the full-length form of the gene; [3]2DS4TOT refers to the total number of 2DS4, *e.g.*, 2DS4DEL and 2DS4WT combined; [4,5]3DL1E4 and 3DL1E9 refers to exon 4 and 9 of the *3DL1* gene respectively. The various KIR genes genotyped are listed in the leftmost column. Genes with S in the name (*e.g.*, KIR2DS4) encode activating KIRs, genes with L in the name (*e.g.*, KIR3DL1) encode inhibiting KIRs and genes with a P in the name (*e.g.*, KIR3DP1) are pseudogenes. Uncorrected *P* value was calculated by the χ^2 test or the Fisher's exact test where appropriate. KIR: Killer immunoglobulin-like receptor; AR: Acute cellular rejection; PSC: Primary sclerosing cholangitis.

Table 12 Killer immunoglobuline-like receptor gene copy number of the recipient and acute cellular rejection after liver transplantation

KIR gene	AR mean copy number (95% CI)	Non-AR mean copy number (95%CI)	*P* value
KIR2DL1	1.69 (1.54-1.84)	1.64 (1.53-1.76)	0.64
KIR2DL2	0.52 (0.34-0.71)	0.53 (0.40-0.65)	0.97
KIR 2DL3	1.50 (1.30-1.70)	1.45 (1.33-1.58)	0.69
KIR 2DL4	2.10 (2.00-2.19)	1.98 (1.91-2.05)	0.06
KIR 2DL5	0.50 (0.27-0.73)	0.63 (0.49-0.78)	0.32
KIR 2DP1	1.71 (1.57-1.86)	1.70 (1.59-1.81)	0.89
KIR 2DS1	0.26 (0.12-0.40)	0.43 (0.33-0.54)	0.08
KIR 2DS2	0.62 (0.30-0.43)	0.54 (0.41-0.66)	0.56
KIR 2DS3	0.29 (0.13-0.44)	0.32 (0.20-0.40)	0.77
KIR 2DS4DEL[1]	1.17 (1.01-1.32)	1.08 (0.94-1.21)	0.45
KIR 2DS4WT[2]	0.55 (0.36-0.73)	0.48 (0.37-0.59)	0.53
KIR 2DS4TOT[3]	1.74 (1.60-1.88)	1.56 (1.45-1.67)	0.06
KIR 2DS5	0.21 (0.08-0.35)	0.32 (0.22-0.42)	0.25
KIR 3DL1E4[4]	1.74 (1.60-1.88)	1.57 (1.46-1.67)	0.08
KIR 3DL1E9[5]	1.74 (1.60-1.88)	1.56 (1.46-1.67)	0.06
KIR 3DL2	-	-	-
KIR 3DL3	-	-	-
KIR 3DP1	-	-	-
KIR 3DS1	0.33 (0.30-0.51)	0.40 (0.30-0.51)	0.47

The various *KIR* genes genotyped are listed in the leftmost column. Genes with S in the name (*e.g.*, KIR2DS4) encode activating KIRs, genes with L in the name (*e.g.*, KIR3DL1) encode inhibiting KIRs and genes with a P in its name (*e.g.*, KIR3DP1) are pseudogenes. Statistical comparison was performed using the independent sample *t* test. [1]2DS4DEL refers to the 22-bp deletion variant of 2DS4; [2]2DS4WT refers to the full-length form of the gene; [3]2DS4TOT refers to the total number of 2DS4, *i.e.*, 2DS4DEL and 2DS4WT combined; [4,5]3DL1E4 and 3DL1E9 refers to exon 4 and 9 of the 3DL1 gene respectively. KIR: Killer immunoglobulin-like receptor; AR: Acute cellular rejection.

of correlated genetic variants within the HLA, including also the HLA-C*07 and DRB1*03 alleles[44,45,47]. The AH8.1 has profound influence on immune function, as also underscored by previously reported associations with a wide range of immune-mediated diseases[44,45]. Results in the present study account for both HLA-B*08 and HLA-C*07 and suggest that the underlying effect represented by these associations localizes in or near these neighboring genes. The dosage effect observed for the PSC associated alleles is also highly supportive of a true effect, *i.e.*, with an AR frequency of 56% and 57%, respectively, in homozygous individuals, as compared with 21% and 18%, respectively, in individuals without HLA-B*08 and HLA-C*07. Importantly, the effect of AH8.1 on AR risk was not restricted to patients with PSC, suggesting that the immunological abnormalities in AH8.1 carriers[45] is of general importance for the pathophysiology in AR. The present results reproduce a previously reported association reported between AH8.1 and AR in liver transplantation[12,48]. There have also been notions of AH8.1 in determining graft survival in kidney transplantation[49]. It is thus timely to explore the immunological basis of this association and the relationship between AR and autoimmunity associations related to the AH8.1 haplotype.

Further support of recipient-related immunogenetic determinants for AR holds true for the DRB1*04 associations, which protect against PSC as well as AR in patients with and without PSC. The AR frequency of individuals without DRB1*04 was 35% (comparable to the overall AR frequency of 28%), while dropping to 12% and 0% for the presence of one and two DRB1*04 alleles, respectively, suggesting a dosage effect of the protective mechanisms represented by the DRB1*04 haplotype. A similar relationship has been noted for type 1 diabetes in kidney transplantation[50]. While DRB1*04 is a strong risk factor for type 1 diabetes[51], type 1 diabetic recipients of diverse ethnicities who carry DRB1*04 experience improved graft survival[50]. A similar shared involvement has also been reported for non-HLA risk loci in autoimmune risk loci, *e.g.*, for the cytotoxic T-lymphocyte antigen 4 (*CTLA4*) gene[52-54]. Most likely the ongoing characterization of shared genetic risk factors between different autoimmune diseases should incorporate recipient factors for allograft-targeted immune reactions[55], for instance by means of screening large recipient populations with and without AR using the Immunochip[43,56].

The precision and homogeneity of the diagnosis of AR is critical for further studies of the genetics of AR along suggested lines. In the present study, great care was taken to include only biopsy proven cases of AR, and the resulting recipient group with AR thus consisted of Banff RAI 3-6 cases with only two exceptions (who showed RAI > 6 in their biopsies). Cases with different etiologies with potentially similar immune manifestations (*e.g.*, ischemia/reperfusion related injuries, biliary pathologies and infections, including early HCV recurrence) were systematically excluded. A multitude of immunological mechanisms are at play in the allograft during the first weeks after liver transplantation, and lack of precision in diagnosis of AR will diminish power considerably in genetic association studies[57,58]. Ideally, data on confounders and rejection characteristics should be prospectively collected, not retrospectively as in the present analysis. Lack of statistical power and phenotypic heterogeneity probably underlies the conflicting results of AR associations for many non-HLA loci investigated [*e.g.*, interleukin 10 (*IL-10*)[59-63],

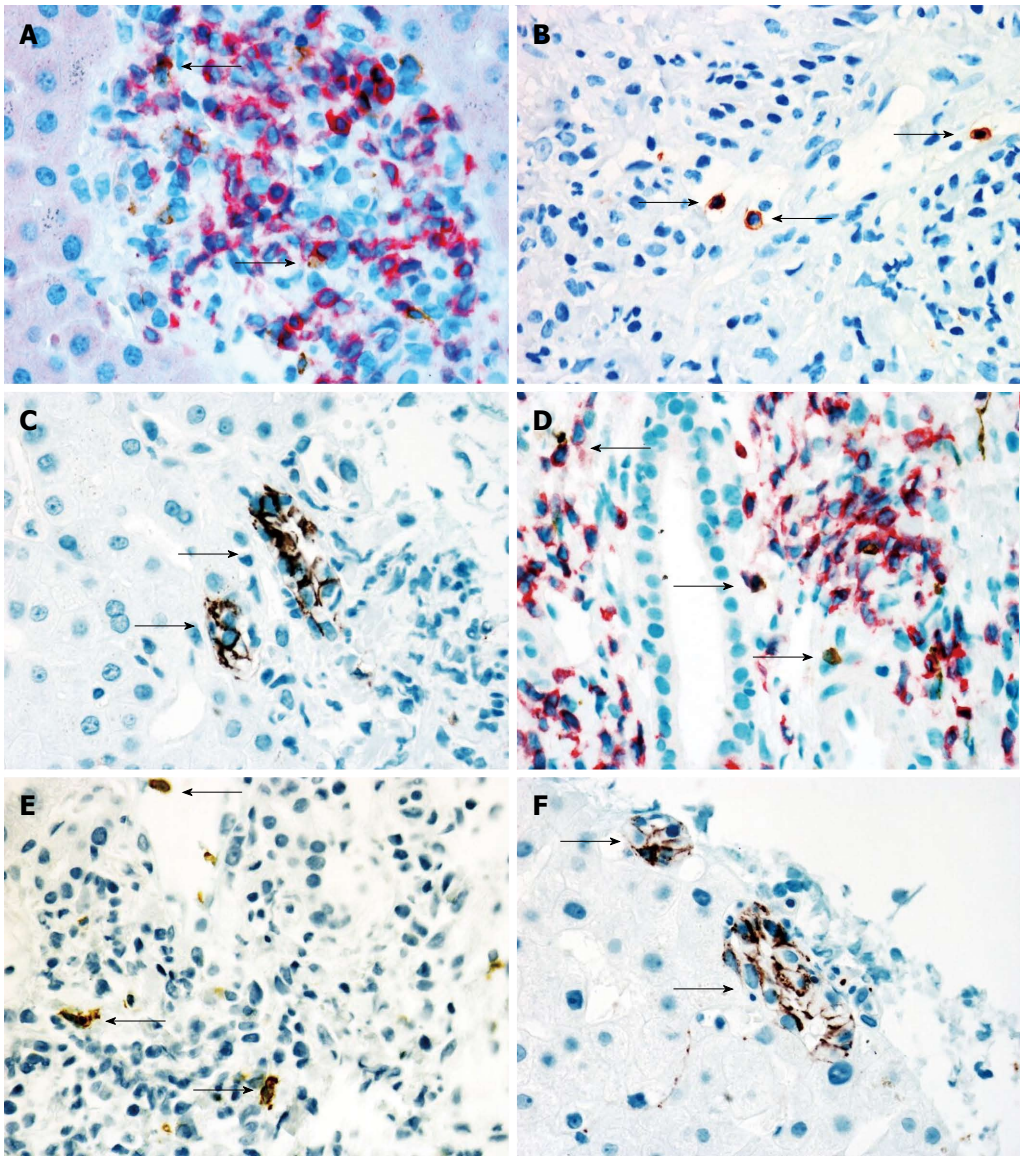

Figure 1 Immunoenzyme stainings in a liver biopsy from a transplanted primary sclerosing cholangitis patient with acute cellular rejection scored according to the Banff criteria as RAI 5 (panels A-C), and from a transplanted control patient (secondary liver metastases) with the same grade of cellular rejection (panels D-F). Panels A and D show double immunoenzyme staining for CD3 (red) and CD56 (brown, arrows) in a portal area. Panels B and E show immunoperoxidase staining for CD57 (brown, arrows) in a portal area. Panels C and F show CD56-staining of bile ducts (arrows). Only scattered CD56- and CD57 positive leucocytes can been seen in both patients (Original magnification × 400).

IL-4 and IL-4 receptor[61,64], *IL-6*[65,66], chemokine receptor 2 (*CCR2*)[67], *CCR5*[67-69], and intercellular adhesion molecule-1 (*ICAM1*)[70,71]]. The present study is also underpowered to detect non-HLA associations for a complex trait like AR[72], but of the same size as those first reporting on what has later shown to be consistent HLA associations in other diseases[46,73]. Similarly, it cannot be excluded that lack of AR association for rare HLA alleles in the present analysis may be due to type II statistical errors.

Some studies have suggested a beneficial effect of HLA matching in LTX[12,48], others have found HLA matching to be detrimental or even exerting a dualistic effect[74]; being beneficial regarding AR, but detrimental to graft/patient survival. In most studies, however, HLA compatibility has not proven to have significant impact on outcome after LTX, and HLA genotype is presently not taken into account in the donor selection protocols. Data in the present population supports the latter conclusion, but also raises the possibility that recipient HLA type may be of consequence for individualized adjustment of the immunosuppressive regimens if findings are confirmed in future studies. Donor-recipient HLA mismatching has previously been reported to be of greater importance in non-autoimmune liver diseases where primary HLA associations do not exist[12]. A more thorough dissection of the HLA-related risk for AR on the side of the recipient only, may thus help clarify HLA determinants on the donor side of relevance to donor-recipient matching.

NK cells have been suggested to engage in the development of liver allograft tolerance[75-77], and animal experiments have suggested that NK cells infiltrate the liver

Table 13 Data showing scarce, but similar numbers of natural killer cells and natural killer T cells in biopsies with equal grade of AR in patients with various primary liver diseases

MNC cell subset[1]	PSC (n = 10)	PBC (n = 10)	Non-imm (n = 10)	P value
CD56 (NK/NKT)	1.6 ± 0.3	1.8 ± 0.4	1.8 ± 0.3	0.9
CD57 (NK/NKT)	5.0 ± 0.9	4.0 ± 0.4	4.6 ± 0.6	0.1
CD3-CD56$^+$ (NK)	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.7
CD3-CD57$^+$ (NK)	0	0	0	-

[1]Mean absolute number of NK/NKT cells/mm^2. Statistics were performed comparing mean value between groups using one-way ANOVA. MNC: Mononuclear cells; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis; Non-imm: Non-immunological disease; NKT: Natural killer T cell; NK: Natural killer.

graft before T lymphocytes[78]. Inspired by work in autoimmune diseases[16], a series of studies have assessed the impact of donor and recipient genetic variation relevant to NK cell activation on risk of AR. While some studies suggest that this interaction may influence the risk of AR after LTX[22,23,41], other studies have failed to support this[19,20]. A major problem in designing the statistical analysis of HLA-KIR interactions, is that biological correlates are poorly defined and the number of potentially relevant interactions is thus high. According to conservative Bonferroni-thresholds for statistical significance, none of the reported associations remain significant. This is also the case for the present dataset. In an analysis accounting for the known biological differences between the HLA-C1 (weak inhibition *via* KIR) and HLA-C2 (strong inhibition *via* KIR)[16], we were unable to detect an influence of the HLA-C2 variant on AR risk[20,21]. Based on previous studies reporting an increased frequency of NK cells in the portal infiltrate of patients with PSC when compared with other liver diseases[79,80], we wanted to examine if similar findings were present in rejection infiltrates in liver transplants. Since the use of NK cell specific monoclonal antibodies was unsuccessful in formalin-fixed material, we used antibodies to CD56 and CD57 to detect putative NK- and NKT cells. The extremely low number of CD56 positive cells in biopsies from patients with AR does not exclude the possibility that these cells contribute to immune an immune reaction primarily driven by the infiltration of T cells. However, the sum of evidence suggests that a genetically determined NK cell hyperreactivity is unlikely to play a major pathogenetic role in AR in LTX.

In conclusion, we detected a significant impact from PSC-associated HLA variants on risk of AR. The findings are similar in PSC and non-PSC recipients and provide important confirmatory support to previous studies in LTX and kidney transplantation, together serving as a basis for further studies of the underlying mechanisms. In addition, we propose that there is a need to query genetic risk of AR along the model of other immune mediated diseases. This with spescial focus on recipients, and aiming for comprehensive genetic coverage by means of genome-wide association studies or targeted genotyping arrays (*e.g.*, the Immunochip) in adequately sized study populations.

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COMMENTS

Background

Donor-recipient matching for human leukocyte antigen (HLA) in liver transplantation is not part of routine clinical practice. Also for other genetic loci, *e.g.*, the killer-immunoglobuline-like receptors (KIR), the clinical importance of donor-recipient matching is unclear. Patients with primary sclerosing cholangitis (PSC) have consistently been reported to exhibit an increased risk of acute cellular rejections (AR) as compared with other indications for liver transplantation. The role of genetic risk factors for PSC in determining risk of AR has not previously been investigated.

Research frontiers

Recent genome-wide association studies have determined multiple robust genetic risk factors for PSC, out of which the strongest localize to the HLA complex on chromosome 6. Genetic risk factors for AR have traditionally been performed along the logic of donor-recipient mismatching, but no consistent findings have so far been made. In particular, recent articles suggest that particular HLA and KIR combinations may be important, but conflicting data also exist.

Innovations and breakthroughs

This is the first study to demonstrate a clear role for HLA variants associated with PSC susceptibility in determining risk of AR. Importantly, findings also apply to patients with other underlying liver diseases. Authors found no significant impact of donor-recipient matching on the risk of AR, neither for HLA nor HLA and KIR combinations.

Applications

Further studies are needed to establish the biological basis for the observed associations. Importantly, the findings suggest that studies querying the role of recipient genetics in AR may provide useful insights into AR pathophysiology. Rather than overly focusing on donor-recipient genetic matching, further studies (*e.g.*, genome-wide association studies) on the recipient side are warranted.

Peer review

The authors offer a valuable contribution to the still debated issue of HLA variants in acute liver rejection development. Further, they have investigated HLA-C and KIR genotypes to demonstrate a lack of association with AR.

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ORIGINAL ARTICLE

Epithelial membrane protein 1 negatively regulates cell growth and metastasis in colorectal carcinoma

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Abstract

AIM: To determine the expression and function of epithelial membrane protein 1 (EMP1) in colorectal carcinoma.

METHODS: Colorectal samples were taken from cancer lesions and adjacent normal tissue in colorectal cancer patients immediately after endoscopic biopsy. A portion of the sample was either fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry or stored in liquid nitrogen for Western blot. In order to determine protein expression of EMP1 in colorectal cancer (n = 63) and normal tissue (n = 31), semi-quantitative immunohistochemistry and Western blot were utilized. For in vitro studies, the human colorectal cancer cell line SW-480 was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Recombinant lentivirus mediated overexpression of EMP1 in SW-480 cells was quantified by real-time reverse transcription-polymerase chain reaction and Western blot. Control SW-480 cells were transfected with an empty vector. To further study the effect of EMP1 overexpression in SW-480 cells, cell proliferation, apoptosis, migration and invasion assays were conducted.

RESULTS: Expression of EMP1 was significantly lower in colorectal cancer tissue than in normal tissue using both immunohistochemistry (39.7% *vs* 90.3% of tissues, $P < 0.05$) and Western blot (0.126 ± 0.022 *vs* 0.632 ± 0.053, $P < 0.05$). The level of EMP1 protein expression was not correlated with gender, age, or tumor location. Decreased expression of EMP1 was significantly correlated with T stage, lymph node metastasis, clinic stage, and histological grade in patients with colorectal cancer ($P < 0.05$). According to Kaplan-Meier analysis, low EMP1 expression correlated significantly with poor overall five-year survival (34.2% *vs* 64.0% survival, $P < 0.05$). SW-480 cells transfected with EMP1 had a lower survival fraction, higher cell apoptosis (12.1% ± 1.3% *vs* 3.1% ± 0.6%, $P < 0.05$), a significant decrease in migration and invasion (124.0 ± 17.0 and 87.0 ± 12.0, respectively *vs* 213.0 ± 29.0 and 178.0 ± 21.0, respectively, $P < 0.05$), higher caspase-9 (0.635 ± 0.063 *vs* 0.315 ± 0.032, $P < 0.05$), and lower VEGFC protein expression (0.229 ± 0.021 *vs* 0.519 ± 0.055, $P < 0.05$) relative to cells not transfected with EMP1.

CONCLUSION: Low EMP1 expression in colorectal cancer is associated with increased disease severity, suggesting that EMP1 may be a negative regulator of colorectal cancer.

Key words: Epithelial membrane protein 1; Colorectal carcinoma; Caspase-9; Vascular endothelial growth factor C; Prognosis

Core tip: Epithelial membrane protein 1 (EMP1) has a known role in tumor development and progression, and its activity is linked to a number of biological processes including proliferation, apoptosis, invasion, and metastasis of colorectal cancer. We detected expression of EMP1 in colorectal carcinoma and analyzed the biological effect of EMP1 overexpression in a colorectal carcinoma cell line. EMP1 expression was decreased in colorectal cancer and its expression correlated significantly with T stage, lymph node metastasis, clinic stage, histological grade, and poor overall survival. Taken together, our findings suggest that EMP1 may play an important role as a negative regulator of colorectal cancer.

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INTRODUCTION

Colorectal cancer is one of the most common malignancies worldwide, and it is a major cause of mortality, with a five-year survival rate of approximately 50%. The main cause of death is metastasis to the liver and lungs, which is present in up to 25% of patients at the time of diagnosis[1,2]. Well-established histopathological factors that influence disease outcome are tumor size, histological type and subtype, the presence of signet ring morphology, the degree of differentiation, the presence of lymphovascular invasion, and lymph node involvement[3,4]. Further understanding of the molecular mechanisms underlying the pathophysiology of metastatic processes will help us not only identify those patients at greatest risk of recurrence but also find novel molecular targets for the development of treatment strategies for colorectal cancer. A preliminary study on the epithelial membrane protein 1 (*EMP1*) gene found that EMP1 is closely linked to tumor development and progression[5,6]. Activation of the *EMP1* gene in particular can prevent tumor proliferation, and it may be a new target for tumor therapy[7,8]. However, to date there is no information available regarding the relationship between EMP1 and colorectal cancer. We studied EMP1 expression in colorectal cancer using immunohistochemistry and Western blot and analyzed the effect of EMP1 overexpression *in vitro* in the colorectal cancer cell line SW-480[9,10].

MATERIALS AND METHODS

Clinical data

All patients enrolled in this study provided informed consent in advance. There were 37 males and 26 females, and they ranged in age from 31 to 78 years, with a median age of 54 years. Of the 63 cases of colorectal cancer, 27 had stages T1 and T2 disease and 36 had stages T3 and T4 disease. Twenty-eight patients did not present with lymph node metastasis (N0), whereas 35 presented with identified lymph node involvement (N+). As for the clinical stage, 25 cases had stage Ⅰ-Ⅱ colorectal cancer and 38 had stage Ⅲ-Ⅳ colorectal cancer. Regarding grade of differentiation, 20 had grade Ⅰ (well differentiated) tumors, and 43 had grade Ⅱ or Ⅲ (moderately to poorly differentiated) tumors. Samples were instantly taken after the endoscopic biopsy, and either fixed in 4% paraformaldehyde solution and embedded in paraffin for immunohistochemistry or stored in liquid nitrogen for Western blot analysis.

Cell culture and gene transfection

Human colorectal cancer SW-480 cells were maintained in RPMI-1640 medium (Gibco BRL, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Gibco BRL). Medium was changed every two to three days; when the cultures reached confluence, the cells were subcultured with 0.25% trypsin and 1% ethylenediaminetetraacetic acid (EDTA). Cells were tested every three months for mycoplasma, and mycoplasma removal agent (MRA) (MP Biomedicals Co. Ltd., Shanghai, China) was used to maintain mycoplasma-free cultures. EMP1 cDNA was cloned into the BamHI and AscI sites of the plenti6/V5-DEST vector (Invitrogen, Carlsbad, CA, United States). After amplification and DNA sequence confirmation, this vector was used to overexpress EMP1 in SW-480 cells. Briefly, SW-480 cells were grown and stably transfected with pLenti6-EMP1 or plenti6/V5-DEST for control using Lipofectamine 2000 (Invitrogen) and grown in Blastidicin (5 μg/mL)-containing RPMI-1640 medium.

Immunohistochemistry

Immunohistochemistry was performed as previously described[11]. Briefly, 4 μm sections were prepared from a paraffin-embedded block and dehydrated, incubated in 3% hydrogen peroxide for 10 min, and incubated in trypsin for 20 min. Sections were blocked with 10% goat serum at room temperature for 20 min and treated with a rabbit anti-human EMP1 polyclonal antibody (1:100; Abcam, Cambridge, United Kingdom) overnight at 4 ℃. After rinsing, sections were treated with biotin-conjugated antibodies (4A Biotech Co. Ltd., Beijing, China) for 20 min, and biotin-immune complexes were identified with a diaminobenzidine (DAB) substrate immunochemistry kit (4A Biotech Co. Ltd.) and hematoxylin stain. Sections were mounted and dehydrated with the coverslip sealed. For the negative control, sections were treated identically except that the primary antibody was replaced with PBS. Two pathologists blinded to patient and tissue status assessed the results. Three slides for each specimen were counted, with five fields of view randomly selected for evaluation per section. EMP1 expression level was based

on the percentage of positive cells and staining intensity. The percentage of positive cells was divided into four levels: 0 points: ≤ 5% of positive cells; 1 point: 5%-25%; 2 points: 25%-50%; and 3 points: > 50%. The intensity of staining was classified as: 0 points: no staining; 1 point: weak staining (light yellow); 2 points: moderate staining (yellowish-brown); and 3 points: strong staining (brown). The final score of EMP1 expression was the product of the EMP1 expression level and intensity, graded as 0 for negative, + for 1-3 points, ++ for 4-6 points, and +++ for 7-9 points.

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from SW-480 cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol[12]. Five hundred nanograms of total RNA was reverse transcribed using TaKaRa Reverse Transcriptase Reagents (TaKaRa, Shiga, Japan). Quantitative real-time (q) reverse transcription-polymerase chain reaction (qRT-PCR) was performed with an ABI Prizm 7300 (Applied Biosystems Inc., Carlsbad, CA, United States) according to the standard protocol for SYBR Premix ExTaq (Perfect Real Time; TakaRa). Primers for EMP1 and β-actin for normalization were as follows: EMP1 sense 5'-CCCTCCTGGTCTTCGTGT, antisense 5'- AATAGCCGTGGTGATA; β-actin sense 5'- ATCGTCCACCGCAAATGCTTCTA, antisense 5'-AGCCATGCCAATCTCATCTTGTT. Thermal cycling conditions were 95 ℃ for 1 min, 95 ℃ for 15 s, and 40 cycles at 60 ℃ for one min. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with SDS 1.3 software (Applied Biosystems Inc.).

Western blot

Western blot was performed as previously described[13]. Samples were lysed in a lysis buffer containing 1% NP-40, 0.1% SDS, 25 mmol/L Hepes, 134 mmol/L NaCl, 1 mmol/L vanadate, 100 mmol/L NaF, and 0.5% Na-deoxycholate. After centrifugation at 12000 r/min for 20 min at 4 ℃, the supernatant was stored at -20 ℃. Protein concentration was detected with the BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing, China). Fifty milligrams of protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. For EMP1, blots were blocked for 2 h with 5% skim milk and incubated overnight at 4 ℃ with rabbit anti-human EMP1 (1:1000), caspase-9 (1:1000; Abcam) and VEGFC (1:1000; Abcam). For β-actin, blots were blocked in 5% nonfat dry milk for 1 h at room temperature and incubated overnight with a mouse anti-β-actin antibody (Sigma, St. Louis, MO, United States) at 4 ℃. After washing, membranes were either incubated with goat anti-mouse fluorescent secondary antibody (1:20000; IRDye800, LI-COR Bioscience, Inc., Lincoln, NE, United States) or DyLight Fluor conjugated goat anti-rabbit secondary antibody (LI-COR Bioscience, Inc.) in the dark for 1 h at room temperature. The blots were scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Bioscience Inc.). Western blot data were quantified by normalizing the signal intensity of each sample to that of β-actin[13].

MTT assay

Cell viability was determined using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described[14]. Briefly, cells were plated into 96-well culture plates at an optimal density of 5×10^3 cells/mL in 200 μL of culture medium per well. After 24-96 h of culture, 20 μL of 5 mg/mL MTT was added to each well and incubated at 37 ℃ for 4 h. The medium was then gently aspirated and 150 μL of dimethyl sulfoxide (DMSO) was added to each well to solubilize the formazan crystals. The optical density of each sample was immediately measured using a microplate reader (BioRad, Hercules, CA, United States) at 570 nm.

Flow cytometry assay

An annexin V-FITC-flow cytometry assay (4A Biotech Co. Ltd.) was used to detect the apoptosis rate in the cells after EMP1 transfection, as previously described[15]. Cells were seeded into 60 mm dishes for 48 h and grown to 70%-75% confluence. After quick detachment from the plate, cells were collected, washed with ice-cold PBS, and resuspended at a cell density of 1×10^6/mL in a binding buffer from the annexin V-FITC apoptosis detection kit (4A Biotech Co. Ltd.). Cells were then stained with 5 μL of annexin V-FITC and 10 μL of propidium iodide (PI, 20 μg/mL). The cells were incubated in the dark at 25 ℃ for 15 min before 10000 cells were analyzed by a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA, United States) and Cellquest software (BD Immunocytometry Systems) for apoptosis rate determination.

Invasion and migration assays

Invasion and migration assays were performed as previously described[16]. For the invasion assay, Costar Transwell 8 μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences, Bedford, MA, United States). Invasion Chambers (BD China, Shanghai, China) were coated with Matrigel, and 10×10^5 cells were added per chamber. Medium supplemented with 10% FBS was used in the lower chamber. For migration assays, the same procedure was used excluding the Matrigel. After 12 h, non-invading cells and media were removed, and cells on the lower surface of the membrane were fixed with polyoxymethylene (Sigma) and stained with 0.1% crystal violet (Sigma) for 0.5 h. Stained cells were counted under a microscope in four randomly selected fields, and the average was used to indicate cell migration and invasion.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (IBM, Chicago, IL, United States), as previously described[17]. For the clinicopathologic features, *P* values were calculated using the χ^2 test. Student's *t* test was used

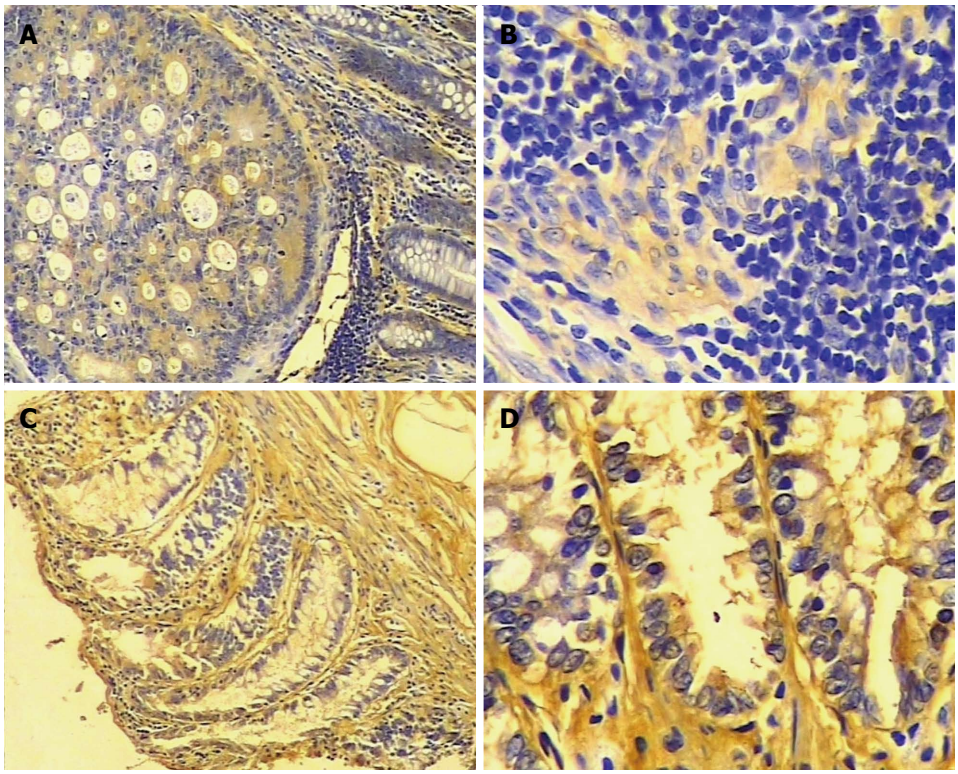

Figure 1 Immunohistochemistry of epithelial membrane protein 1 protein in colorectal carcinoma and adjacent normal tissue. A and B: Representative sample of colorectal carcinoma (A: SP × 100, B: SP × 400). There is little staining for epithelial membrane protein 1 (EMP1); C and D: normal colorectal tissue (C: SP × 100, D: SP × 400). There is intense yellow and yellow-brown staining of EMP1.

Table 1 Expression of epithelial membrane protein 1 in colorectal cancer tissue and normal colorectal tissue

Group	Case	Expression of epithelial membrane protein 1					
		-	+	+ +	+ + +	χ^2	*P* value
Normal tissue	31	3	6	11	12	25.239	0
Cancer tissue	63	38	10	8	7		

to analyze the difference between groups. Survival distributions were estimated by the Kaplan-Meier method and compared using the log-rank test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

EMP1 protein expression in colorectal cancer and normal tissues

EMP1 staining in colorectal cancer tissue was negative or weak relative to normal adjacent colorectal tissues that exhibited light yellow to brown staining. EMP1 expression was significantly lower ($P < 0.05$) in colorectal cancer tissue (expressed in 39.7%, 25/63) than in normal tissue (expressed in 90.3%, 28/31) (Figure 1 and Table 1). Western blot analysis showed that the expression of EMP1 in cancer lesions was significantly less than that in adjacent normal tissue (0.126 ± 0.022 and 0.632 ± 0.053, respectively, $P < 0.05$) (Figure 2). The expression of EMP1 negatively correlated with T stage, lymph node metastasis, clinical stage and pathological differentiation ($P < 0.05$), regardless of age, gender, and tumor location (P

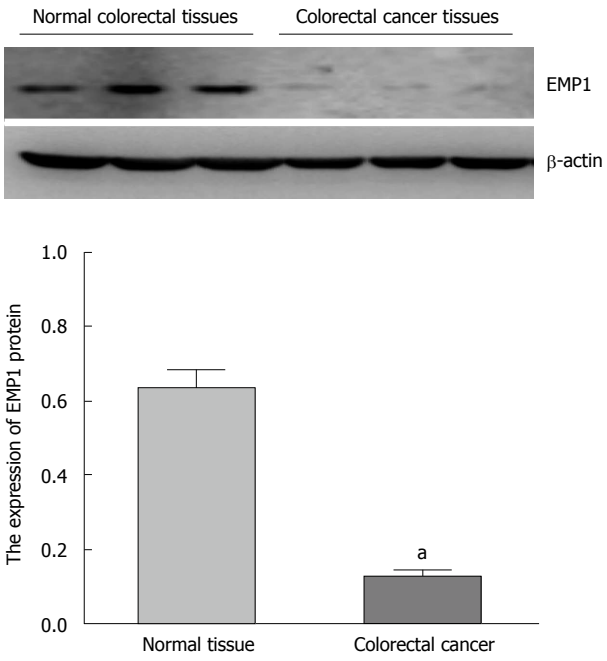

Figure 2 Epithelial membrane protein 1 protein in colorectal carcinoma and normal tissue detected by Western blot. Upper panel, representative blots of normal (left) and cancer colorectal (right) tissue. Lower panel, summary of all samples. Epithelial membrane protein 1 (EMP1) levels are significantly less in colorectal cancer relative to control, $^aP < 0.05$.

Table 2 Relationship between epithelial membrane protein 1 expression and clinical characteristics of colorectal cancer

Variable	Case	Expression of epithelial membrane protein 1			
		-	+-+++	χ^2	*P* value
Sex					
Male	37	21	16	0.199	0.656
Female	26	15	9		
Age (yr)					
≤ 60	46	27	19	0.187	0.665
> 60	17	11	6		
Tumor location					
Colon cancer	35	19	16	1.197	0.274
Rectal cancer	28	19	9		
Tumor invasion					
T1 + T2	27	11	16	7.566	0.006
T3 + T4	36	27	9		
Lymph node metastasis					
N0	28	11	17	9.314	0.002
N+	35	27	8		
Clinical stage					
Ⅰ-Ⅱ	25	9	16	10.240	0.001
Ⅲ-Ⅳ	38	29	9		
Histological grade					
Ⅰ	20	8	12	5.054	0.025
Ⅱ-Ⅲ	43	30	13		

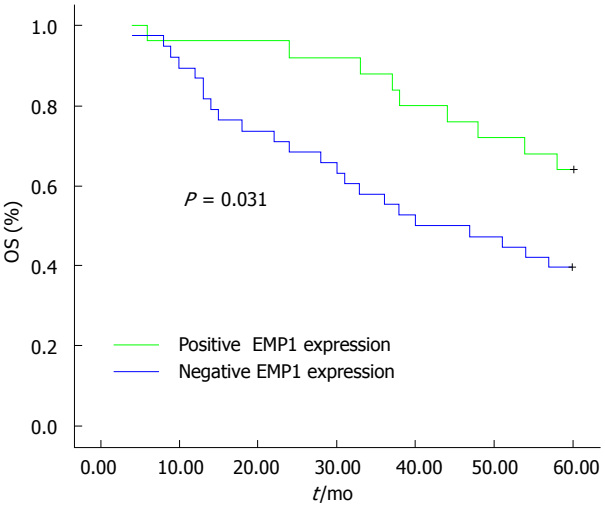

Figure 3 Relationship between epithelial membrane protein 1 expression and five-year survival in colorectal carcinoma by Kaplan-Meier analysis. Overall survival is higher in epithelial membrane protein 1 (EMP1)-positive patients relative to EMP1-negative patients.

> 0.05, Table 2).

EMP1 expression and prognosis

Patients were followed for 60 mo for survival analysis. At the end of the study in 2012, 29 of 63 patients had survived. Patients were divided into two groups according to expression level of EMP1. Of the 25 patients with positive levels of EMP1 expression, 16 were still alive, yielding a survival rate of 64.0%. Of the 38 patients with undetectable levels of EMP1 expression, only 13 were still alive, yielding a survival rate of 34.2%. Patients with high levels of EMP1 had a significantly higher five-year survival rate than those with low levels of EMP1 ($P < 0.05$) (Figure 3).

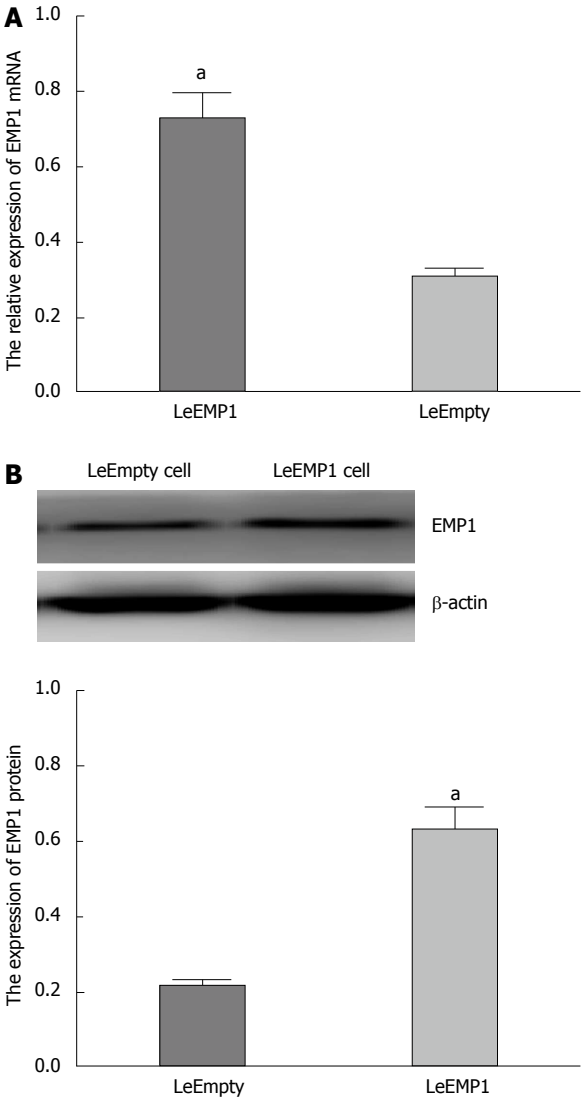

Figure 4 Expression and identification of the epithelial membrane protein 1 gene. A: Reverse transcription-polymerase chain reaction for epithelial membrane protein 1 (EMP1) in LeEmpty cells *vs* LeEMP1 cells; B: Sample Western blots for EMP1 and actin (loading control) in LeEmpty and LeEMP1 cells (top). Summary of Western blot data for EMP1 protein expression (bottom), $^aP < 0.05$ *vs* LeEMP1.

Stable transfection of EMP1 cDNA in colorectal cancer cells

SW-480 cells stably transfected with EMP1 overexpressed EMP1 (named as LeEMP1 cells). Control SW-480 cells were transfected with an empty vector (named as LeEmpty cells). The expression of EMP1 mRNA and protein was significantly elevated in LeEMP1 cells relative to control cells ($P < 0.05$). EMP1 mRNA levels detected by RT-PCR were significantly higher in LeEMP1 cells (0.729 ± 0.066) than in LeEmpty cells (0.305 ± 0.028) ($P < 0.05$; Figure 4A). Western blot analysis found that the level of immunoreactive protein was significantly higher in EMP1 transfected cells (0.631 ± 0.060) relative to controls cells

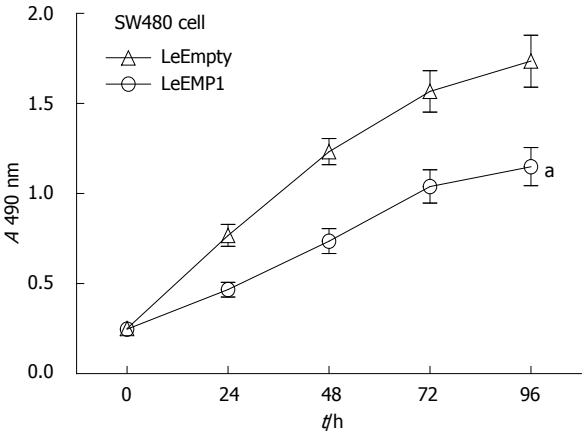

Figure 5 Effects of epithelial membrane protein 1 overexpression on cell proliferation. MTT assay time-course for LeEmpty and LeEMP1 cells. Cells overexpressing EMP1 have a significantly decreased rate of proliferation relative to control cells, $^{a}P < 0.05$. EMP1: Epithelial membrane protein 1.

(0.213 ± 0.018) ($P < 0.05$; Figure 4B).

Effects of EMP1 overexpression on colorectal cancer cells

Next, we assessed the effect of EMP1 expression on the regulation of colorectal cancer cell viability. MTT assay showed that relative proliferative capacity of LeEMP1 cells grew significantly slower at 24, 48, 72, and 96 h relative to LeEmpty cells ($P < 0.05$; Figure 5). Meanwhile, there was a significant increase in the early apoptosis rate in LeEMP1 cells (12.1% ± 1.3%) relative to control cells (3.1% ± 0.6%) ($P < 0.05$; Figure 6). SW-480 cells transfected with EMP1 or empty vector were transferred to transwell chambers or Matrigel-coated transwell chambers to evaluate the effect of EMP1 on cell invasion potential. Overexpression of EMP1 significantly decreased cell migration and invasion of SW-480 cells (124.0 ± 17.0 and 87.0 ± 12.0, respectively) relative to control cells (213.0 ± 29.0 and 178.0 ± 21.0, respectively) ($P < 0.05$; Figure 7).

To further study the mechanisms by which EMP1 inhibited colorectal cancer cell proliferation, apoptosis, migration, and invasion, we analyzed the expression of two proteins with critical roles in these processes, caspase-9 and VEGFC. Western blot analysis revealed that overexpression of EMP1 in SW-480 cells significantly upregulated caspase-9 protein expression (0.635 ± 0.063) relative to control cells (0.315 ± 0.032) ($P < 0.05$; Figure 8). In contrast, the level of VEGFC protein expression was significantly lower in SW-480 cells overexpressing EMP1 (0.229 ± 0.021) than in control cells (0.519 ± 0.055) ($P < 0.05$; Figure 8).

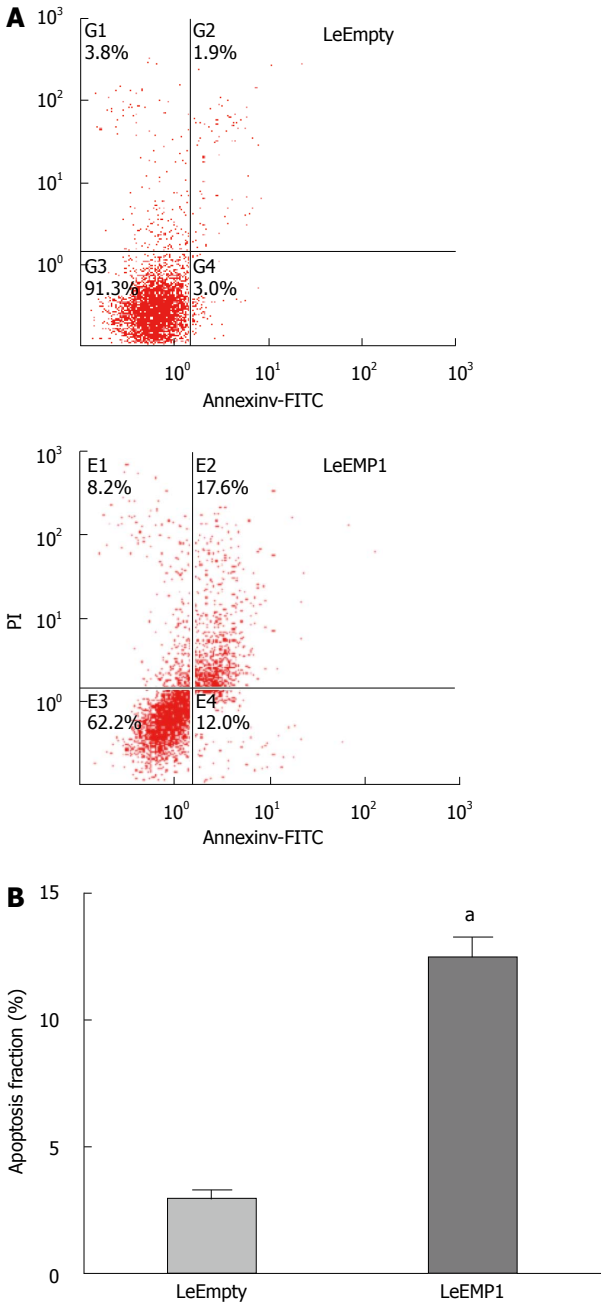

Figure 6 Effects of epithelial membrane protein 1 overexpression on cell apoptosis. A: Cells were stained with 5 μL annexin V-FITC and 10 μL PI (20 μg/mL). Samples were acquired on a FACScan flow cytometer and 10000 cells analyzed with Cellquest software; B: Colorectal cancer cells overexpressing epithelial membrane protein 1 (LeEMP1) exhibit significantly more apoptosis than empty vector transfected cells (LeEmpty), $^{a}P < 0.05$

DISCUSSION

Several studies have shown that the *EMP1* gene is expressed in a number of normal tissues[7,18-23]. In this study, we localized and quantified for the first time EMP1 protein expression in colorectal cancer tissue and normal colorectal tissue using immunohistochemistry and immunoblotting. EMP1 protein levels were significantly lower in colorectal carcinoma than in normal tissue, and EMP1 protein levels correlated with T stage, lymph node metastasis, and clinical stage of colorectal cancer. Since dedifferentiation is a hallmark of tumor cells, our findings suggest that a decline in EMP1 level is a factor in the development and progression of colorectal cancer. In a study evaluating several types of human breast cancer cells with different metastatic characteristics, *EMP1* gene expression was correlated with cell invasion and other properties of metastasis[24]. *EMP1* gene expression was

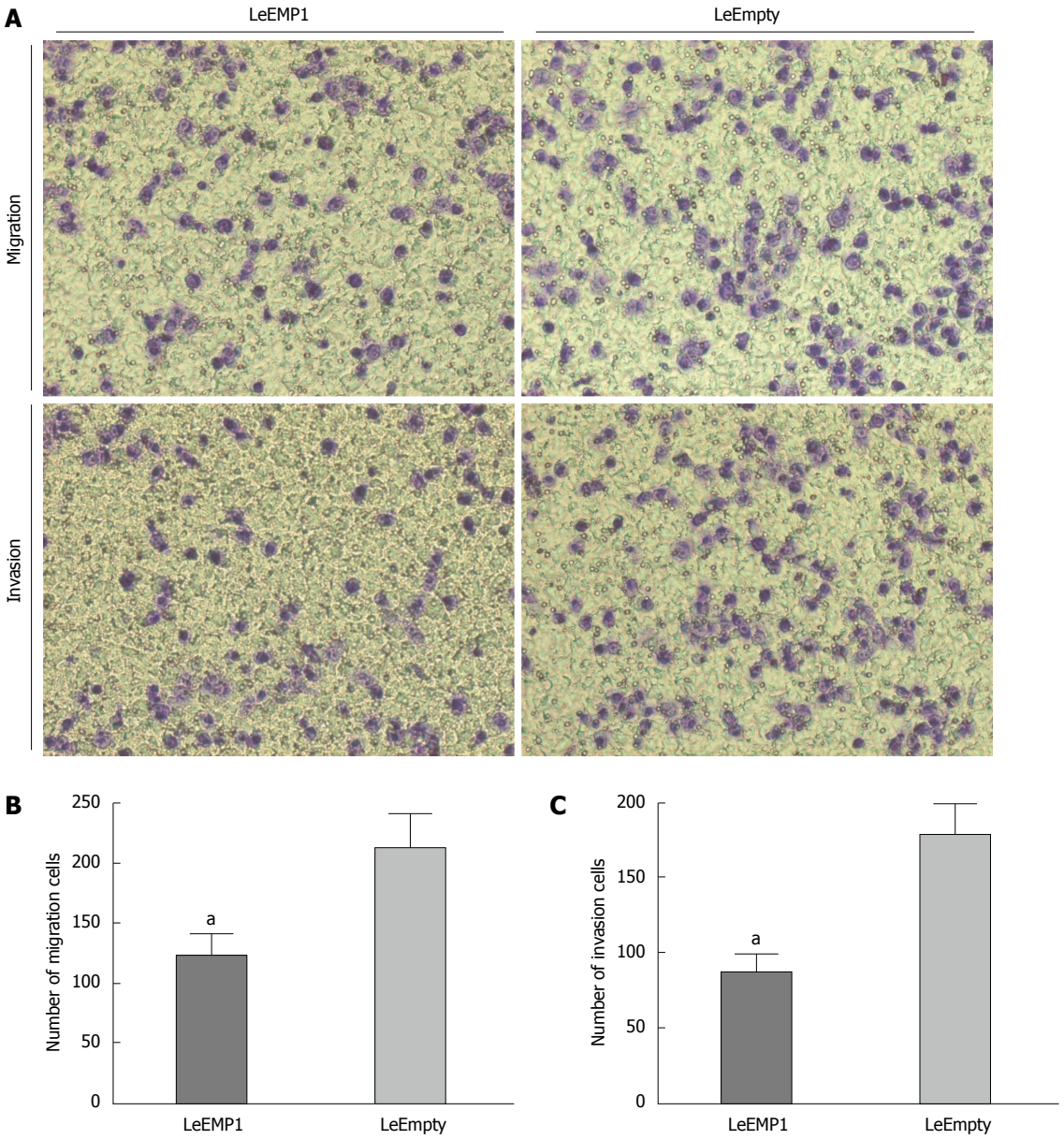

Figure 7 Effects of epithelial membrane protein 1 overexpression on cell migration and invasion. A: Histological sections of cell migration and invasion in LeEmpty and LeEMP1 cells; B: The number of migrating cells is significantly greater in LeEmpty cells than in LeEMP1 cells; C: Number of invading cells is greater in LeEmpty than in LeEMP1 transfected cells, $^{a}P < 0.05$. EMP1: Epithelial membrane protein 1.

down-regulated in oral squamous cell carcinoma and this down-regulation was correlated with lymph node metastasis[25]. Therefore, the EMP1 gene may be an important factor for the regulation of cell signaling, cell communication, and adhesion[26].

Currently an effective treatment paradigm for colorectal cancer is extended surgical resection of the lesion, accompanied by chemotherapy and/or radiotherapy before and after surgery. However, the five-year survival rate with this strategy is only 40%[27,28]. Therefore, efforts should be directed toward early detection of colorectal cancer and the refinement of individual based treatment strategies. Conventional treatment and prognosis of colorectal cancer rely mainly on TNM classification[29]. This system is subjective and not informative for early colorectal cancer, and offers limited information about disease severity, prognosis, and response to treatment. Early detection of colorectal cancer is the most effective way to improve survival[30]. Using survival analysis, we found that EMP1 expression-positive patients had a significantly higher five-year overall survival rate than patients with undetectable EMP1 expression. Thus, combining information from the TNM classification system and EMP1 expression scores may provide valuable information for clinicians regarding prognosis, prediction of disease severity, and selection of treatment regimens.

Furthermore, in vitro experiments demonstrated for the first time that colorectal cancer cells with high EMP1 expression had significantly weakened proliferation, significantly increased apoptosis, and markedly reduced

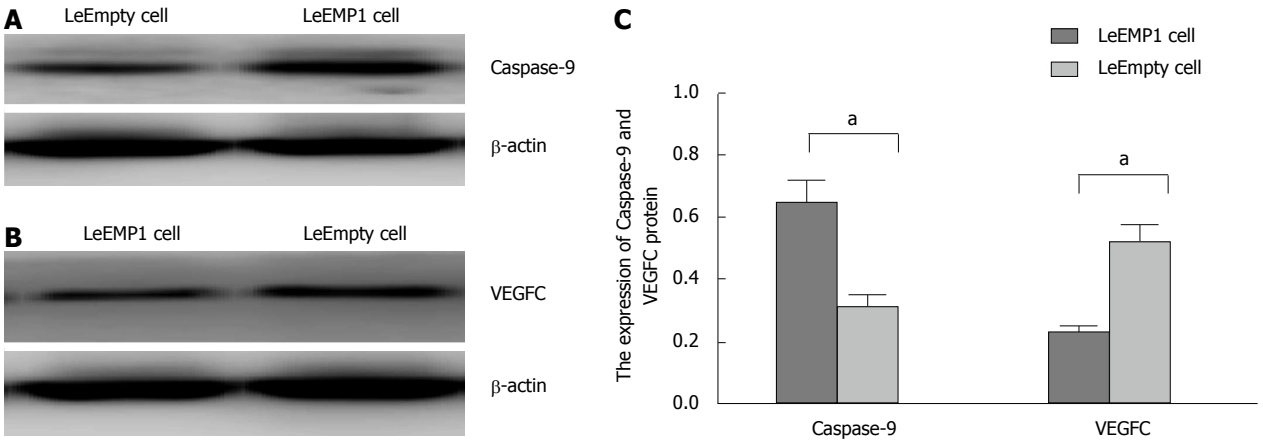

Figure 8 Effects of epithelial membrane protein 1 overexpression on caspase-9 and vascular endothelial growth factor-factor C expression. A: Sample blot for caspase-9 and actin (loading control) of colorectal cancer cells transfected with LeEMP1 and LeEmpty; B: Sample blot for vascular endothelial growth factor-factor C (VEGFC) and actin; C: Quantification of caspase-9 and VEGFC expression in LeEMP1 and LeEmpty cells. $^{a}P < 0.05$ beteween the two groups.

caspase-9 and VEGFC protein levels. Previously, overexpression of EMP1 in an esophageal cancer cell line slowed esophageal cancer cell growth and yielded fewer S-phase cells and more G_1-phase cells[26]. Together with our findings, these data suggest that low levels of EMP1 affect cellular processes that are abnormally regulated in cancer. Mitochondria are not only the site of cellular respiration and oxidative phosphorylation, but also the regulation center of apoptosis. Cytochrome C released from mitochondria to the cytoplasm associates with apoptotic protease activating factor (Apaf-1) to form a multiservice complex in the presence of deoxyribonucleotide triphosphate (dNTP)[31]. This complex interacts with pro-caspase-9 to form an apoptosome and, following dimerization, results in autoactivation of caspase-9. This activated caspase-9 stimulates other caspases, such as caspase-3 and caspase-7, culminating in apoptosis *via* signaling cascades[32-34]. We found in this study that high expression of EMP1 is associated with significantly higher expression of caspase-9 protein, implicating a mitochondrial apoptosis pathway in EMP1-induced apoptosis.

VEGF is a member of the platelet-derived growth factor (PDGF) family and is the most important vascular endothelial growth-stimulating factor during tumor angiogenesis. VEGFC is a recently identified member of the VEGF family, which promotes the proliferation of endothelial cells, increases vascular permeability, and functions as a key factor in tumor angiogenesis, invasion, and metastasis[35,36]. We found in this study that overexpression of EMP1 is associated with a significant decrease in VEGFC expression. This finding suggests that EMP1 may inhibit tumor angiogenesis by suppressing VEGFC expression and hence tumor metastasis.

In summary, we demonstrated that EMP1 protein levels were significantly reduced in colorectal carcinoma and were associated with T stage, lymph node metastasis, clinical stage, and cell differentiation. EMP1 is involved in a number of biological processes including proliferation, apoptosis, invasion, and metastasis of colorectal cancer. Given the complexity of carcinogenesis, further research is needed to understand the molecular mechanism underlying EMP1 regulation of this process. Our findings identify a novel potential therapeutic target for colorectal cancer and suggest that EMP1 may be a reliable biomarker for prognosis of colorectal cancer.

COMMENTS

Background

Colorectal carcinoma remains one of the leading causes of global cancer mortality. Further understanding of the molecular mechanisms underlying the metastatic process will help us to identify those at highest risk of recurrence and to identify novel tumor targets to prevent disease progression. Although epithelial membrane protein 1 (EMP1) has been implicated in tumor development and progression, its role in colorectal carcinoma remains unknown.

Research frontiers

EMP1 protein is believed to be in the same protein family as peripheral myelin 22 (PMP22) and shares high sequence homology with PMP22 (approximately 40%). EMP1 is also found in the liver, heart, lung, bone, muscle, kidney, spleen, prostate, testis, ovary, placenta and thymus. EMP1 is highly expressed in undifferentiated embryonic stem cells and lowly expressed in differentiated adult cells, prolonging the transition of Schwann cells from G-phase to S + G_2/M-phase. It has been suggested that this membrane glycoprotein family is closely related to cell proliferation and differentiation.

Innovations and breakthroughs

The authors report for the first time that EMP1 protein levels were significantly reduced in colorectal carcinoma. EMP1 expression was associated with T stage, lymph node metastasis, clinical stage, and cell differentiation. EMP1 is involved in a number of biological processes including proliferation, apoptosis, invasion, and metastasis of colorectal cancer, suggesting that EMP1 may play important roles as a negative regulator of these processes in colorectal cancer cells.

Applications

The results of this study contribute to a better understanding of the association between the loss of EMP1 in colorectal cancer and tumorigenesis and progression of this cancer. The findings identify a novel therapeutic target for colorectal cancer and suggest that EMP1 may be a reliable biomarker for the prognosis of colorectal cancer.

Terminology

Lentiviral vectors derived from the human immunodeficiency virus (HIV-1) have become major tools for gene delivery in mammalian cells. The primary advantage of using lentiviral vectors is the ability to mediate potent transduction and stable expression into dividing and non-dividing cells both *in vitro* and *in vivo*. Lentiviral vectors are typically produced in HEK 293T cells. Essential lentiviral (*HIV-1*) genes must be expressed in these cells to allow the generation of lenti-

viral particles.

Peer review

Sun *et al* analyzed the expression, clinical significance of EMP1 in colorectal carcinoma and the biological effect in a colorectal carcinoma cell line by EMP1 overexpression using cell and molecular biological and biochemical techniques. The study was well designed and contributed to the understanding that EMP1 protein levels were significantly lower in colorectal carcinoma than in normal tissue.

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RESEARCH REPORT

Immunohistochemical assessment of NY-ESO-1 expression in esophageal adenocarcinoma resection specimens

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Abstract

AIM: To assess NY-ESO-1 expression in a cohort of esophageal adenocarcinomas.

METHODS: A retrospective search of our tissue archive for esophageal resection specimens containing esophageal adenocarcinoma was performed, for cases which had previously been reported for diagnostic purposes, using the systematised nomenclature of human and veterinary medicine coding system. Original haematoxylin and eosin stained sections were reviewed, using light microscopy, to confirm classification and tumour differentiation. A total of 27 adenocarcinoma resection specimens were then assessed using immunohistochemistry for NY-ESO-1 expression: 4 well differentiated, 14 moderately differentiated, 4 moderate-poorly differentiated, and 5 poorly differentiated.

RESULTS: Four out of a total of 27 cases of esophageal adenocarcinoma examined (15%) displayed diffuse cytoplasmic and nuclear expression for NY-ESO-1. They displayed a heterogeneous and mosaic-type pattern of diffuse staining. Diffuse cytoplasmic staining was not identified in any of these structures: stroma, normal squamous epithelium, normal submucosal gland and duct, Barrett's esophagus (goblet cell), Barrett's esophagus (non-goblet cell) and high grade glandular dysplasia. All adenocarcinomas showed an unexpected dot-type pattern of staining at nuclear, paranuclear and cytoplasmic locations. Similar dot-type staining, with varying frequency and size of dots, was observed on examination of Barrett's metaplasia, esophageal submucosal gland acini and the large bowel negative control, predominantly at the crypt base. Furthermore, a prominent pattern of apical (luminal) cytoplasmic dot-type staining was observed in some cases of Barrett's metaplasia and also adenocarcinoma. A further morphological finding of interest was noted on examination of haematoxylin and eosin stained sections, as aggregates of lymphocytes were consistently noted to surround submucosal glands.

CONCLUSION: We have demonstrated for the first time NY-ESO-1 expression by esophageal adenocarcinomas, Barrett's metaplasia and normal tissues other than germ cells.

Key words: Esophageal adenocarcinoma; Immunohistochemistry; NY-ESO-1; Stem cells; Vesicle trafficking

Core tip: NY-ESO-1 is a cancer-testis antigen of particular interest, as it displays exceptional immunogenicity

- hence, it is an attractive candidate for cancer vaccine therapy. To our knowledge, we have demonstrated for the first time, using immunohistochemistry, strong and diffuse nuclear and cytoplasmic expression for NY-ESO-1 in a cohort of esophageal adenocarcinoma cases. We have also demonstrated NY-ESO-1 expression in normal tissues other than germ cells, albeit as dot-positivity, indicating shared protein expression and association between primitive germ cells and somatic cells. We further relate our findings to proposed locations of stem cells in the esophagus and large bowel.

Hayes SJ, Hng KN, Clark P, Thistlethwaite F, Hawkins RE, Ang Y. Immunohistochemical assessment of NY-ESO-1 expression in esophageal adenocarcinoma resection specimens. *World J Gastroenterol* 2014; 20(14): 4011-4016 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4011.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4011

INTRODUCTION

Cancer-testis antigens (CT Ag) are a subset of cancer antigens which are widely expressed by a variety of malignant tumours. NY-ESO-1 is a CT Ag of particular interest, as it displays exceptional immunogenicity, making it an attractive candidate for cancer vaccine therapy[1]. Previous studies have reported it to be expressed by spermatogonia, oogonia and placenta.

NY-ESO-1 is expressed in varying frequency by many types of malignant tumour: immunohistochemical (IHC) studies for example have shown expression in 7/130 (6%) of endometrial carcinomas; 13/52 (25%) of non small cell carcinomas of the lung; and 18/22 (82%) of neuroblastomas. Two separate IHC studies of squamous carcinoma of the esophagus have shown expression in 44/213 (21%) and 18/56 (32%) of cases[1].

NY-ESO-1 has also been shown to be expressed by mesenchymal stem cells, using immunofluorescence performed on cultured cells derived from bone marrow aspirates and fetuses[2].

To our knowledge, its expression in esophageal adenocarcinoma (EAC) has not been investigated. Hence, further study is warranted, as this malignancy represents an attractive candidate for cancer vaccine treatment.

EAC has shown an increasing incidence over the past three decades, and it has a poor prognosis, with a 5 year survival rate of less than 10%[3,4]. Barrett's esophagus (BE) is a metaplastic pre-malignant lesion for EAC, and both BE and EAC are considered of stem cell derivation, in accordance with the cancer stem cell theory[3,5-7].

In this study, we investigate NY-ESO-1 expression by IHC in a cohort of 27 EAC resection specimens; these specimens contained other esophageal tissue structures, including putative stem cell compartments, and this provides an additional opportunity to explore the proposition made by Cronwright *et al*[2], of NY-ESO-1 representing a stem cell marker.

The location of esophageal stem cells remains open to debate, but an emerging theory proposes a location within the submucosal gland/duct unit[8-11]. A further suggested location is the basal compartment of the interpapillary layer of the squamous epithelium[12]. Indeed, it may be the case that more than one population of progenitor cells exists within the esophagus[6].

MATERIALS AND METHODS

Subjects and specimen collection

The study was approved by the National Research Ethics Service (NRES Committee North West-Cheshire, Rec reference: 11/NW/0632). A retrospective search was made of the tissue archive at Salford Royal NHS Foundation Trust for esophageal resection specimens containing EAC. This search was made over a 7 month period, using the systematised nomenclature of human and veterinary medicine coding system, for cases which had previously been reported for diagnostic purposes. This search revealed a total of 27 EAC cases: 4 well differentiated; 14 moderately differentiated; 4 moderate-poorly differentiated and 5 poorly differentiated. Original sections, stained with haematoxylin and eosin (HE), were reviewed using light microscopy, by a histopathologist with a gastrointestinal interest (Hayes SJ), to confirm classification and tumour differentiation. One representative section of each tumour was selected for IHC, and these sections were noted to include the following additional components: 27/27 stroma; 22/27 normal squamous epithelium; 7/27 normal esophageal duct; 10/27 normal submucosal gland; 9/27 BE (goblet cell); 3/27 BE (non-goblet cell, cardiac type); 1/27 BE (non-goblet cell, fundic-type); 1/27 high grade glandular dysplasia.

Immunohistochemistry

All tissues had been fixed in neutral buffered formaldehyde and processed into paraffin wax by standard histological methods. Further 3 μm thick sections were cut and placed on adhesive coated slides (Snowcoat, Surgipath Europe). Sections were dried overnight at 60 ℃.

IHC staining was performed using a Leica Bond Max immunostainer. Antigen retrieval was done for 25 min at high pH using Bond epitope retrieval solution 2, (Leica AR9640). NY-ESO-1 was identified using anti-NY-ESO-1, clone E978 (Invitrogen), diluted 1 + 400 for 30 min. Antibody binding was visualised using a Bond polymer refine detection kit (Leica DS9800) according to the manufacturer recommendations. The sections were then dehydrated, cleared and cover-slipped.

Sections of normal adult testis were used as a positive control. Normal colon, thyroid gland and omentum were selected as negative control sections.

RESULTS

Out of a total of 27 cases of EAC examined, 4 cases (15%) displayed diffuse cytoplasmic and nuclear ex-

pression for NY-ESO-1: 2 moderately differentiated; 1 moderately-poorly differentiated; 1 poorly differentiated. They displayed a heterogeneous and mosaic-type pattern of diffuse staining (Figure 1A). Furthermore, the pattern of staining seen in all 4 cases was noted to have a rather granular pattern (Figure 1B).

As noted in the methodology, the cancer cases selected for assessment of NY-ESO-1 expression also contained the following tissue components: stroma; normal squamous epithelium; normal submucosal gland/duct; BE (goblet cell); BE (non-goblet cell); and high grade glandular dysplasia.

Diffuse cytoplasmic staining was not identified in any of these structures (P value < 0.005, χ^2 test). However, an interesting dot-type pattern of expression for NY-ESO-1 was detected in all of these structures, with varying frequency and sizes of dots. Individual cells contained single or multiple dots, which were present at different cellular locations (nuclear, paranuclear and cytoplasmic).

Occasional extremely small and barely discernible speckles of NY-ESO-1 expression were identified at the following locations and frequency: stroma (17/27); basal aspect of squamous epithelium (12/22); normal duct (5/7); BE, non-goblet cell-fundic type (1/1).

A dot-type pattern of staining comprising of more frequent and predominantly larger (medium sized dots), was seen at the following locations: normal submucosal gland (7/10, Figure 1C); BE, goblet cell (9/9); BE, non-goblet cell-antral type (3/3). These larger dots were also located at nuclear, paranuclear and cytoplasmic locations.

A further interesting pattern of staining was seen on examination of 8/9 BE cases (goblet cell type) and in 1/3 of the cases of BE (antral type); these cases demonstrated a prominent pattern of apical (luminal) cytoplasmic dot-type staining (Figure 1D).

The largest and most frequent dots were identified in cases of high grade glandular dysplasia (HGD) (1/1) and EAC (27/27) (Figure 1E). Of the 4 cases of EAC demonstrating a diffuse heterogeneous pattern of expression, the areas of tumour with an absence of diffuse cytoplasmic expression did show a pattern of dot-type expression. One case of EAC which appeared moderately differentiated showed prominent cytoplasmic dot-type staining, with dots seen to be closely located and coalescing in places (perhaps representing incipient diffuse staining). Two cases of EAC (one well differentiated and one moderately-poorly differentiated) showed a prominent apical cytoplasmic pattern of staining, similar to that seen in BE (Figure 1F).

Some variation in dot size was identified in EAC (*i.e.*, as well as large dots being present, some medium sized dots were also identified). Variation was also demonstrated in the frequency of dots seen; most cases of EAC showed a high frequency of dot-type staining, but 2 cases (one well differentiated and the other poorly differentiated) showed only a scanty pattern of dot-type expression.

A further morphological finding of interest was noted on examination of HE stained sections; aggregates of lymphocytes were consistently seen to surround submucosal glands in the tissue sections examined.

The positive control sections of adult testis showed cytoplasmic and nuclear basal staining of spermatogonia and primary spermatocytes, in accordance with that reported by previous studies[2] (Figure 1G).

Thyroid gland and omentum, used as negative control sections consistently showed an absence of staining for NY-ESO-1. However, sections of large bowel used as a negative control displayed occasional unexpected medium-sized dot-type staining. A total of 9/11 large bowel negative control sections showed dot-expression for NY-ESO-1 (nuclear, paranuclear and cytoplasmic locations). The total number of dots observed was 26, of which 19 (73%) were located at the crypt base and 7 (27%) were located at a mid-crypt location.

It was noted that one basal crypt showed a particularly interesting pattern of dot-expression, containing a nuclear dot, a paranuclear dot and two cytoplasmic dots (both of which were located in alignment with and apparently corresponding to the paranuclear dot) (Figure 1H).

DISCUSSION

To our knowledge, we have demonstrated for the first time, using IHC methodology, strong and diffuse nuclear and cytoplasmic expression for NY-ESO-1 in a cohort of EAC cases (15%); this is comparable to the frequencies of expression reported in esophageal squamous carcinoma (21% and 32%)[1]. Our findings indicate that at least a sub-set of EACs may be responsive to cancer vaccine treatment.

In this study, diffuse expression was noted in tumours that appeared moderately, moderately-poorly and poorly differentiated. None of the well differentiated tumours examined showed evidence of diffuse staining. Therefore, these findings suggest an association between NY-ESO-1 expression in EAC and a more poorly differentiated phenotype.

Furthermore, all of the tumours demonstrating diffuse expression showed evidence of heterogeneity, with an interesting mosaic-type pattern (Figure 1A). We speculate this to relate to a general signalling defect, perceived by localised groups of cells over-expressing NY-ESO-1, or alternatively, localised amplification of stem cell activity. As cancer-testis antigen expression is thought to be regulated epigenetically, it is uncertain whether the heterogeneous pattern of NY-ESO-1 expression observed in this and previous studies are a stable trait, or whether it varies over time (*i.e.*, those cells not expressing the protein at one time point may demonstrate expression at a separate time point)[11]. As such, it is at present uncertain whether heterogeneity of expression would affect response to cancer vaccine therapy.

This study describes a varying frequency and size of dot-type staining (nuclear, paranuclear and cytoplasmic) within normal esophageal tissue compartments, BE, HGD and EAC. Occasional dot-type expression for NY-ESO-1, involving extremely small speckles of expression, were identified by the stroma, esophageal squamous epi-

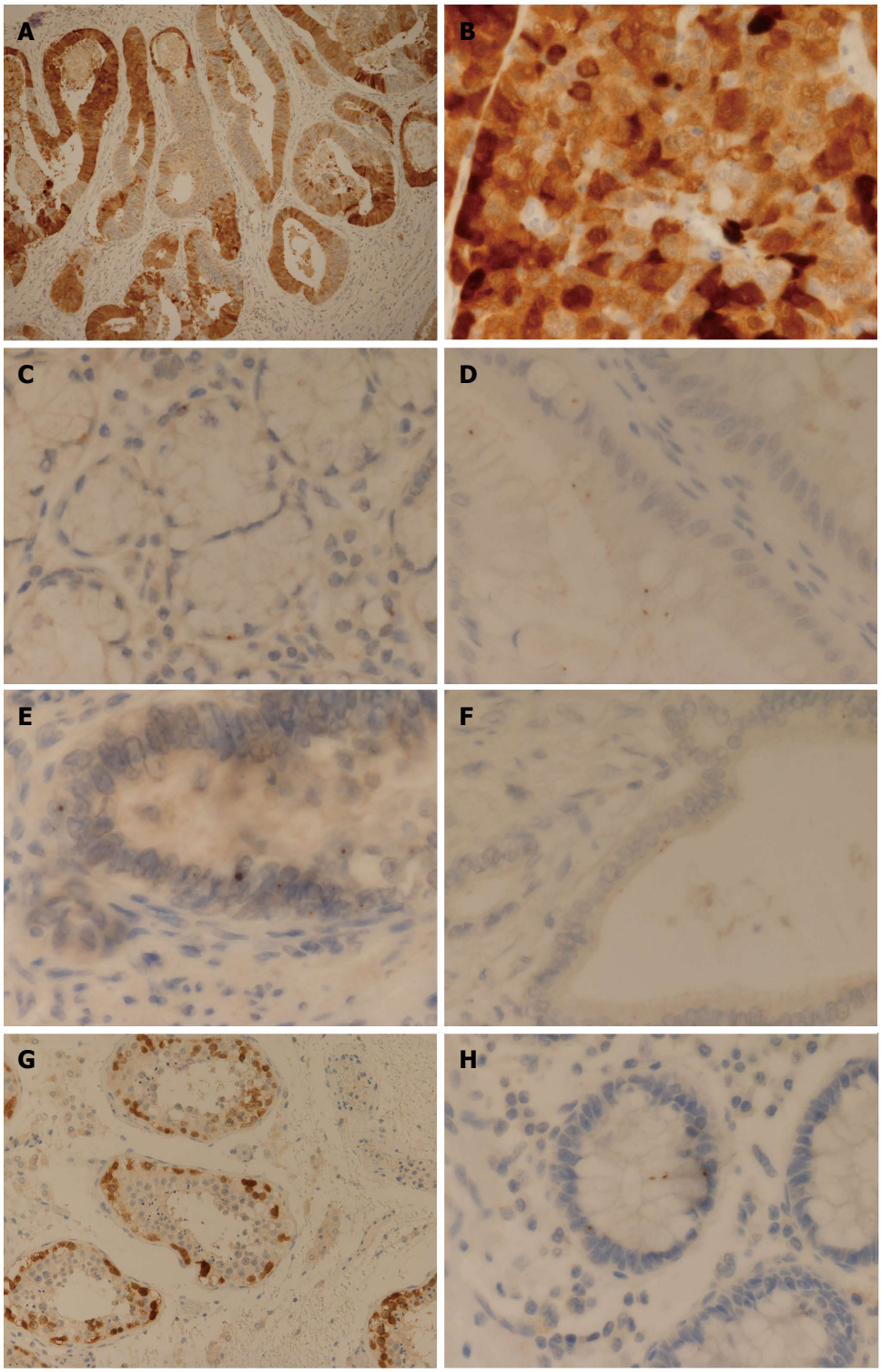

Figure 1 Immunohistochemistry images. A: Esophageal adenocarcinoma (EAC) demonstrating diffuse, heterogeneous and mosaic-type expression for NY-ESO-1 (immunohistochemistry, IHC × 10); B: EAC showing a diffuse and granular pattern of cytoplasmic NY-ESO-1 expression (IHC × 40); C: Esophageal submucosal gland demonstrating dot-type expression for NY-ESO-1 (IHC × 60); D: Barrett's esophagus of intestinal-type showing apical cytoplasmic dot-type expression for NY-ESO-1 (IHC × 60); E: EAC showing nuclear dot-type expression for NY-ESO-1 (IHC × 60); F: Well differentiated EAC with apical cytoplasmic dot-type expression for NY-ESO-1 (IHC × 60); G: Positive control (testis) demonstrating diffuse NY-ESO-1 expression which is restricted to primitive germ cells (IHC × 20); H: Negative control (colon) with dot-type pattern at nuclear, paranuclear and cytoplasmic locations, involving a basal crypt. Two of the cytoplasmic dots show alignment and apparent relationship to the paranuclear dot (IHC × 60).

thelium (towards its base), submucosal gland ducts and BE (fundic-type). These findings are of uncertain significance, as these speckles were barely identifiable using light microscopy.

Larger and more frequent dot-type expression was identified by submucosal glands, BE (goblet cell-type)

and BE (antral-type). The largest dots were seen in HGD and EAC.

A pattern of dot-type staining on examination of IHC markers at multiple cellular locations is highly unusual. The possibility has been considered of it representing a staining artefact. However, NY-ESO-1 expression was noted to be strong and crisp in the tissue sections examined, including the positive control, and there was an absence of background staining; in our experience, these features indicate the experimental technique to have worked appropriately. As such, we consider the possibility of staining artefact to be unlikely and the unusual pattern of expression seen suggests a biological process of great interest. It is more typical in histopathological practice, for instance, to observe dot-type staining for IHC markers at a single location, such as paranuclear staining for CK20 in Merkel cell carcinoma.

It is uncertain as to what these dots actually represent, and this will require further investigation. They do not with any certainty represent the structure of an organelle, but perhaps more likely relate to aggregates of protein.

A further purpose of this study was to investigate putative esophageal stem cell compartments. Stem cells within the gastrointestinal tract have proven histologically elusive, making it necessary to define IHC markers to identify them. Some previous success has been achieved in defining candidate stem cell markers in the large bowel, such as LgR5 and CD133[5,13]; at this location, for example, investigation using LgR5 has helped to clarify the presence of multiple stem cells, residing at the crypt base[13]. In relation to these findings, we have described unexpected dot-type staining for NY-ESO-1, predominantly located at the bases of crypts, on examination of colonic tissue used as a negative control; this adds further support to the proposition made by Cronwright *et al*[2], of NY-ESO-1 representing a stem cell marker.

Stem cell expression for NY-ESO-1 could be perceived as a potential handicap, considering that NY-ESO-1 is currently being targeted by cancer vaccine treatment; however, vaccine treatment targeting NY-ESO-1 has been reported to be well tolerated, and this may relate to an important distinction between the dot expression and diffuse expression for NY-ESO-1[1].

The only normal tissue compartment examined which contained a larger and more frequent dot-type pattern of NY-ESO-1 expression was the esophageal submucosal gland unit. As NY-ESO-1 has been proposed to represent a stem cell marker, this would support the emerging theory, postulating the submucosal gland to represent an esophageal stem cell compartment[2]. Of further interest, Cronwright *et al*[2] described NY-ESO-1 to localise to nucleoli-like structures in mesenchymal stem cells, representing a similar pattern to the nuclear dot-type staining observed in our study.

Furthermore, we have described the presence of lymphoid aggregates, probably related to a process of gastric reflux, which were consistently noted to show tropism for submucosal gland units; this inflammatory cell infiltrate may have a role in activation of stem cells within the submucosal gland. Additional supportive evidence for NY-ESO-1 representing a stem cell marker is provided by the presence of significantly sized dot-type staining for NY-ESO-1 involving BE, HGD and EAC, which are all considered of stem cell derivation[5-7].

Although BE showed expression for NY-ESO-1 at different cellular locations, expression was prominently demonstrated within the cytoplasm, often at an apical and luminal aspect. A similar pattern of expression was also noted on examination of EAC cases; a possible explanation for this is that NY-ESO-1 may be involved in a process of vesicle trafficking in BE and EAC, with paracrine function associated with exocytosis into the esophageal lumen. This process is thought to be involved in cellular communication, whereby microvesicles have been suggested to cause epigenetic reprogramming of target cells[14]. Such a process could potentially contribute to the documented phenomenon of field cancerization associated with BE and EAC[15]. Alternatively, vesicles in this instance may have a role in protein storage, or transport to another part of the cell, such as the nucleus, depending on their function,

A limitation of this study is related to the rather difficult and subjective assessment of dot size; this was addressed by the involvement of a pathologist in this study (Hayes SJ) who has experience in morphological assessment. Further studies should aim for further refinement in the accuracy of dot size assessment.

In conclusion, this study demonstrates, using IHC methodology, diffuse expression for NY-ESO-1 in a proportion of EAC cases. Furthermore, it demonstrates for the first time, NY-ESO-1 to be expressed in normal tissues other than germ cells, albeit as dot-positivity. If further study could demonstrate dot-type expression in other normal tissues, then this would provide correlation with previously described, but unexplained, low levels of NY-ESO-1 mRNA expression observed in normal tissues[16,17]. Our findings provide supportive evidence that NY-ESO-1 represents a stem cell marker, which in turn indicates shared protein expression and association between primitive germ cells and somatic stem cells. This study also provides supportive evidence for both the esophageal submucosal gland unit and the crypt base of large bowel to represent stem cell compartments.

Finally, we advocate the merit of future studies with an aim to further clarify the precise nature and function of NY-ESO-1 expression in normal, metaplastic and neoplastic tissues.

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COMMENTS

Background

Many genes which are expressed in the testis are also expressed in cancer,

and they are commonly termed cancer-testis antigens. NY-ESO-1 is a cancer-testis antigen of particular interest, as it displays exceptional immunogenicity, making it an attractive candidate for cancer vaccine therapy.

Research frontiers

Esophageal adenocarcinoma is recognised as a difficult to treat malignancy with a poor prognosis and, as such, it represents an attractive candidate for cancer vaccine therapy. To our knowledge, NY-ESO-1 expression in esophageal adenocarcinoma (EAC) has not been investigated. Hence, further study is warranted, as this malignancy represents an attractive candidate for cancer vaccine treatment.

Innovations and breakthroughs

Authors have demonstrated for the first time, using immunohistochemistry, expression for NY-ESO-1 in a cohort of esophageal adenocarcinoma cases. They have also described a novel dot-type pattern of expression for NY-ESO-1 in normal, metaplastic and neoplastic tissues. Their findings provide supportive evidence that NY-ESO-1 represents a stem cell marker, which in turn indicates shared protein expression and association between primitive germ cells and somatic stem cells. This work and other previous studies also provide supportive evidence for both the esophageal submucosal gland unit and the crypt base of large bowel to represent stem cell compartments.

Applications

Esophageal adenocarcinoma expressing NY-ESO-1 represents a candidate for cancer vaccine therapy.

Terminology

Cancer-testis antigens: a subset of cancer antigens which are widely expressed by a variety of malignant tumours. NY-ESO-1: a CT Ag of particular interest which was initially discovered using serological identification of antigens by recombinant expression cloning (SEREX) methodology. Immunohistochemistry: a standardized method of using specific epitope(s) and antibody binding technology, to localize and study expression of particular antigen(s)/marker(s). Stem cells: pluripotent cell lineage capable of potential differentiations or transdifferentiations.

Peer review

This is a novel study, demonstrating NY-ESO-1 expression in esophageal adenocarcinomas, which has utility with regard to further immunotherapy treatment. Further studies are advocated in order to clarify the significance of the described dot-type expression in normal, metaplastic and neoplastic tissues.

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RESEARCH REPORT

Positive predictors for gastroesophageal reflux disease and the therapeutic response to proton-pump inhibitors

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Abstract

AIM: To identify objective and subjective predictors for the reliable diagnosis of gastroesophageal reflux disease (GERD) and the response to proton pump inhibitor (PPI) therapy.

METHODS: Retrospectively, 683 consecutive patients suspected for GERD who underwent pH-metry/impedance measurement (pH/MII) were analyzed. All patients had previously undergone standard PPI treatment (*e.g.*, pantoprazole 40 mg/d or comparable). Four hundred sixty patients were at least 10 d off PPIs (group A), whereas 223 patients were analyzed during their ongoing PPI therapy (group B). In addition, all patients completed a standardized symptom- and lifestyle-based questionnaire, including the therapeutic response to previous PPI trials on a 10-point scale. Uni- and multivariance analyses were performed to identify criteria associated with positive therapeutic response to PPIs.

RESULTS: In group A, positive predictors (PPs) for response in empirical PPI trials were typical GERD symptoms (heartburn and regurgitation), a positive symptom index (SI) and pathological results in pH/MII, along with atypical symptoms, including hoarseness and fullness. In group B, regular alcohol consumption was associated with the therapeutic response. The PPs for pathological results in pH/MII in group A included positive SI, male gender, obesity, heartburn and regurgitation. In group B, the PPs were positive SI and vomiting. Analyzing for positive SI, the PPs were pathological pH and/or MII, heartburn regurgitation, fullness, nausea and vomiting in group A and pathological pH and/or MII in group B.

CONCLUSION: Anamnestic parameters (gender, obesity, alcohol) can predict PPI responses. In non-obese, female patients with non-typical reflux symptoms, pH/MII should be considered instead of empirical PPIs.

Key words: Gastroesophageal reflux; Gastroesophageal reflux disease; Non-erosive reflux disease; Impedance pH measurement; Follow-up; Therapy; Proton pump inhibitor

Core tip: The response rates to proton pump inhibitors in reflux disease vary. Empirical proton pump inhibitor therapy poses a substantial economic burden. Positive predictors of the therapeutic response are necessary. This study provides the highest number of reflux patients. Anamnestic, objective and subjective parameters predicting the therapeutic response were evaluated.

Becker V, Grotz S, Schlag C, Nennstiel S, Beitz A, Haller B, Schmid RM, Meining A, Bajbouj M. Positive predictors for gastroesophageal reflux disease and the therapeutic response to proton-pump inhibitors. *World J Gastroenterol* 2014; 20(14): 4017-4024 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4017.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4017

INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders worldwide[1-4]. In western countries, approximately 40% of the adult population suffers occasionally from reflux symptoms; approximately 20% report symptoms at least once per week[5,6]. Symptoms of GERD are subdivided into typical/esophageal (heartburn, regurgitation) and atypical/extraesophageal symptoms (chronic cough, hoarseness, recurrent sinusitis, globus sensation in the throat, burning feeling on the tongue, dental erosion, fullness)[7]. Symptom overlap is common[8].

The most effective therapeutic approaches for GERD symptoms are proton pump inhibitor (PPI) trials[9,10]. Therapy response rates for PPIs vary but are more satisfactory in patients with erosive reflux disease (ERD) and typical reflux symptoms. However, the data are conflicting in patients with non-erosive reflux disease (NERD) and/or atypical/extraesophageal symptoms and functional disorders (FD)[11]. Nonetheless, the discrimination between NERD and FD is challenging. Distinguishing between patients who adequately respond to PPIs and those who remain symptomatic is a matter of debate. PH/MII is considered a useful tool for answering this question[12,13]. This technique enables the reliable detection and quantification of non-acidic, weakly acidic and acid reflux episodes in the esophagus with high sensitivity for all types of reflux episodes[14]. Combined esophageal pH/MII monitoring patterns can also discriminate between NERD and FD[15,16].

Separate from the classifications, the ultimate clinical goal in all patients is most likely symptom relief after PPI therapy. Knowing the reliable and specific anamnestic findings and/or parameters of pH/MII for predicting symptom relief might lead to more selective PPI therapies than empiric PPI tests. The benefit of PPI tests is controversially discussed[17,18]. These trials pose an extensive economic burden and contribute substantially to overall health-care expenditures[19]. However, with higher response rates to PPIs, unnecessary treatment may be avoided, which could result in tremendous savings in resources.

Therefore, the aim of our study was to identify objective and subjective parameters that might predict the therapeutic response to PPIs in patients with suspected GERD for guiding therapy, particularly in the primary-care setting.

MATERIALS AND METHODS

Patients

This retrospective study included 683 consecutive patients who underwent pH/MII for suspected GERD between January 2007 and December 2011 at the Technische Universität München, Munich, Germany. The indication to perform pH/MII was suspected GERD with typical and/or atypical reflux symptoms. The inclusion criteria were a previous standard PPI trial [*e.g.*, pantoprazole 40 mg/d (or comparable) within the last 6 mo], with positive or negative symptom relief, and endoscopy of the upper gastrointestinal tract within the last 12 mo to exclude malignancy. The exclusion criteria were a history of previous gastric or esophageal surgery or severe esophageal motility disorders. Informed consent for data evaluation was obtained from all patients. The study was approved by the Ethics Commission of the Technischre Universität München.

Before pH/MII, all patients were asked to complete a lifestyle- and symptom-based questionnaire to query their personal characteristics (weight, height, age, relevant disorders, smoking and drinking habits) and symptoms (heartburn, regurgitation, globus sensations, burning feeling on the tongue, chronic cough, hoarseness, fullness, nausea, vomiting and halitosis) on a 10-point scale. A subjective response to PPI therapy was defined as a symptom reduction of at least 3 points on the 10-point scale.

PH/MII monitoring

Combined pH/MII was performed using an ambulatory, multi-channel, intra-luminal impedance system consisting of a portable data logger and a combined pH-impedance catheter (Tecnomatix ZAN S 61 C 01 E, Sandhill Scientific, Highlands Ranch, CO, United States). Six impedance electrodes and a distal antimony-pH probe were placed at pre-defined spots on this catheter (3.0, 5.0, 7.0, 9.0, 15.0 and 17.0 cm; pH probe, 5.0 cm). The catheter was inserted with the antimony pH probe located 5 cm above the manometrically defined lower esophageal sphincter. Data recording was performed for 22-24 h. The stored data were then uploaded to a personal computer and individually analyzed using a commercially available software system (BioView, Sandhill Scientific). Gastroesophageal reflux detected from impedance changes was defined based on previous reports[20,21].

Reflux episodes were defined as either acidic or non-acidic, when a retrograde bolus movement was detected via impedance and the pH value was below or above 4, respectively. Furthermore, the content of the reflux episode was characterized according to its composition (gas, fluid or mixed). Following the suggested reference values published by Shay *et al*[22] and Zerbibb *et al*[14], the MII was considered pathological when more than 73 fluid and/or mixed reflux episodes occurred in the esophagus over the 22- to 24-h recording period. The esophageal pH measurement was considered pathological when the

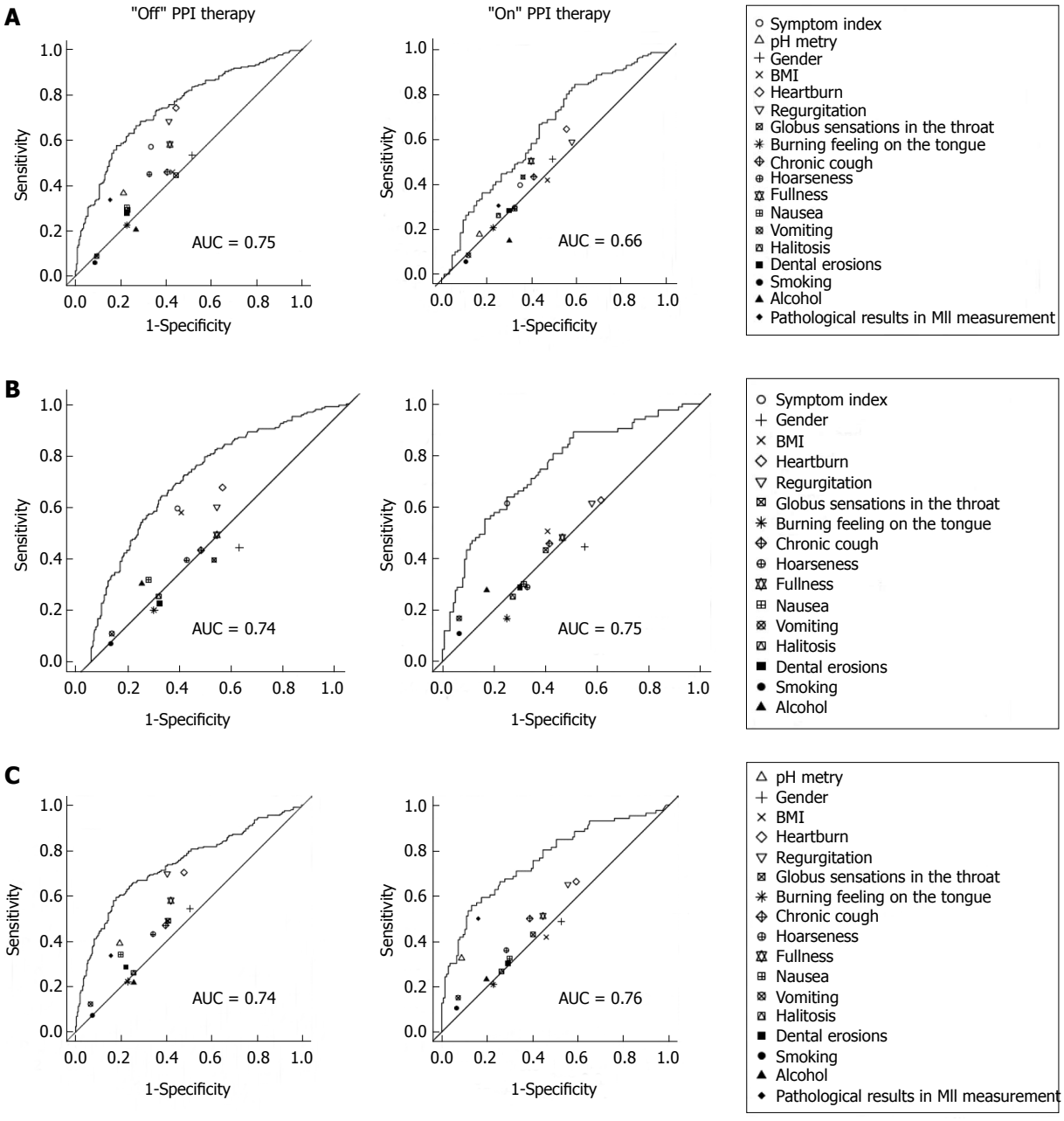

Figure 1 Receiver operating characteristics analysis of the respective symptoms in association with the response to pump inhibitor therapys and the pathological parameters from pH-metry/impedance measurement. A: Response to standard PPI therapy; B: Parameters associated with pathological parameters in pH/ MII; C: Parameters associated with pathological SI.

period during which the pH was below 4 was more than 4% overall. Meals were excluded from the analysis.

The patients were asked to indicate their predominant symptoms during the course of the measurements to assess the symptom index (SI). The SI was assessed as positive when at least half of each specific symptom' s duration was associated with reflux episodes over a 5-minute interval.

Alcohol consumption was defined as equal to or more than 15 g per day (on more than 3 d per week); cigarette consumption was defined as equal to or more than 10 cigarettes per day.

Statistical analysis

For the qualitative data, absolute and relative frequencies are presented; for the quantitative data, medians are shown. To determine the association between the relevant measures and the study endpoints, possible predictor variables were dichotomized, and the sensitivities, specificities, positive and negative predictive values and odds ratios were estimated. To test for associations, continuity-corrected chi-squared tests were performed. Multiple logistic regression models, including all relevant measures as the independent variables, were fit to the data. Goodness of fit was assessed by a receiver operating characteristics

Table 1 Response to proton pump inhibitor therapy

Parameters	Sensitivity	Specificity	PPV	NPV	OR (univ)	*P*val (univ)	OR (mult)	*P*val (mult)
Group A								
SI	0.57	0.67	0.58	0.66	2.66	< 0.001	1.67	0.021
pH-metry	0.37	0.79	0.58	0.61	2.15	< 0.001	1.63	0.055
Gender	0.53	0.49	0.45	0.56	1.07	0.772	0.97	0.9
BMI	0.46	0.57	0.46	0.57	1.13	0.567	0.85	0.465
Heartburn	0.74	0.56	0.57	0.73	3.6	< 0.001	2.31	0.001
Regurgitation	0.68	0.59	0.57	0.7	3.08	< 0.001	1.64	0.059
Globus sensation	0.44	0.56	0.45	0.55	1	1	0.8	0.315
Burning tongue	0.22	0.77	0.44	0.55	0.98	1	0.67	0.129
Coughing	0.46	0.6	0.48	0.58	1.25	0.279	1.05	0.849
Hoarseness	0.45	0.67	0.53	0.6	1.69	0.009	1.59	0.054
Fullness	0.58	0.58	0.53	0.63	1.95	0.001	1.52	0.067
Nausea	0.3	0.77	0.52	0.58	1.47	0.087	0.83	0.505
Vomiting	0.09	0.91	0.43	0.55	0.93	0.944	0.65	0.275
Halitosis	0.29	0.77	0.5	0.57	1.37	0.166	1.13	0.619
Bile taste	0.28	0.77	0.5	0.57	1.31	0.255	0.9	0.67
Imp path	0.34	0.85	0.64	0.61	2.81	< 0.001	1.99	0.009
Smoking	0.06	0.91	0.35	0.55	0.66	0.342	0.65	0.905
Alcohol	0.2	0.73	0.38	0.53	0.71	0.152	0.77	0.313
Group B								
SI	0.41	0.66	0.67	0.39	1.35	0.373	1.18	0.627
Ph-metry	0.19	0.84	0.67	0.38	1.26	0.662	1.09	0.848
Gender	0.52	0.51	0.65	0.38	1.16	0.693	0.88	0.689
BMI	0.43	0.54	0.62	0.35	0.88	0.759	0.84	0.569
Heartburn	0.66	0.45	0.67	0.43	1.59	0.134	1.88	0.086
Regurgitation	0.6	0.43	0.64	0.38	1.13	0.769	0.71	0.345
Globus sensation	0.45	0.65	0.68	0.4	1.48	0.222	1.33	0.367
Burning tongue	0.22	0.78	0.63	0.37	1	1	0.84	0.636
Coughing	0.45	0.6	0.66	0.39	1.2	0.614	1.21	0.575
Hoarseness	0.31	0.68	0.63	0.37	0.98	1	0.75	0.406
Fullness	0.52	0.61	0.7	0.42	1.68	0.089	2	0.033
Nausea	0.3	0.68	0.62	0.36	0.95	0.969	0.62	0.205
Vomiting	0.1	0.89	0.61	0.36	0.89	0.984	0.88	0.8
Halitosis	0.28	0.76	0.66	0.38	1.19	0.707	1.21	0.595
Bile taste	0.3	0.71	0.64	0.37	1.03	1	0.88	0.717
Imp path	0.32	0.76	0.69	0.39	1.45	0.299	1.38	0.381
Smoking	0.07	0.9	0.56	0.36	0.71	0.653	0.75	0.595
Alcohol	0.16	0.71	0.49	0.33	0.47	0.034	0.43	0.03

PPV: Positive predictive value; NPV: Negative predictive value; SI: Symptom index.

(ROC) analysis investigating the relationship between the predicted probabilities and the true value of the dependent variable of the logistic regression model. To illustrate the additional information obtained from the multiple regression model compared with the univariate results, sensitivities and specificities for all relevant measures were drawn in the ROC plot. All statistical tests were performed based on a two-sided level of significance (α = 5%). The statistical software programs SPSS version 20 and R version 2.15.1 were used for the analyses.

RESULTS

Six hundred eighty-three (329 male) patients who fulfilled the inclusion criteria were identified retrospectively by analysis of our pH/MII database [median age, 54.8 years; median body mass index (BMI), 24.7]. During the pH/MII, 460 patients were off (group A) and 223 patients were on (group B) PPI therapy.

First, the therapeutic response to standard PPI therapy was analyzed (Table 1). In group A, the positive predicting parameters for the therapeutic response were SI, pathological results from the pH-metry, heartburn, regurgitation, hoarseness, fullness and pathological results from the MII measurement. In group B, alcohol consumption was associated with the therapeutic response.

Second, the findings associated with pathological parameters in the pH/MII were analyzed (Table 2). In group A, the positive predicting parameters that correlated with pathological results from the pH/MII were SI, male gender, increased BMI index, heartburn, regurgitation, nausea and alcohol consumption. In group B, SI and vomiting were associated with pathological results from the pH/MII.

Third, parameters associated with a pathological SI were analyzed (Table 3). In group A, the positive predicting parameters for a pathological SI were pathological results from the pH measurement, heartburn, regurgitation, fullness, nausea, vomiting and pathological results from the impedance measurement. In group B, pathological results from the pH and MII measurements were significantly associated with a positive SI. Furthermore,

Table 2 Parameters associated with pathological results from pH-metry/impedance measurement

Parameters	Sensitivity	Specificity	PPC	NPV	OR (univ)	*P*val (univ)	OR (mult)	*P*val (mult)
Group A								
SI	0.59	0.66	0.54	0.71	2.92	< 0.001	2.74	< 0.001
Gender	0.44	0.42	0.34	0.53	0.59	0.008	0.63	0.045
BMI	0.58	0.65	0.53	0.7	2.56	< 0.001	2.36	< 0.001
Heartburn	0.68	0.49	0.47	0.69	2.01	0.001	1.88	0.017
Regurgitation	0.6	0.51	0.45	0.66	1.58	0.023	1.06	0.816
Globus sensation	0.39	0.52	0.36	0.56	0.72	0.102	0.66	0.064
Burning tongue	0.2	0.76	0.36	0.58	0.78	0.325	0.73	0.228
Coughing	0.43	0.57	0.41	0.6	1.03	0.958	0.99	0.978
Hoarseness	0.39	0.63	0.42	0.61	1.1	0.678	1.21	0.432
Fullness	0.49	0.51	0.4	0.6	1.02	0.998	0.83	0.424
Nausea	0.32	0.78	0.49	0.63	1.64	0.027	1.63	0.078
Vomiting	0.11	0.92	0.48	0.6	1.39	0.389	1.06	0.876
Halitosis	0.25	0.74	0.39	0.59	0.96	0.938	0.93	0.783
Bile taste	0.23	0.73	0.36	0.59	0.81	0.41	0.69	0.154
Smoking	0.07	0.92	0.38	0.6	0.91	0.95	0.81	0.606
Alcohol	0.3	0.8	0.51	0.63	1.78	0.012	1.9	0.012
Group B								
SI	0.61	0.75	0.59	0.77	4.78	< 0.001	4.091	< 0.001
Gender	0.45	0.45	0.32	0.58	0.66	0.172	0.84	0.625
BMI	0.51	0.59	0.42	0.67	1.49	0.195	1.73	0.097
Heartburn	0.63	0.39	0.38	0.63	1.05	0.969	0.95	0.906
Regurgitation	0.61	0.42	0.39	0.65	1.16	0.699	1.1	0.795
Globus sensation	0.43	0.6	0.39	0.64	1.15	0.723	1.31	0.428
Burning tongue	0.17	0.75	0.29	0.6	0.61	0.211	0.55	0.163
Coughing	0.46	0.59	0.4	0.65	1.19	0.621	1.2	0.608
Hoarseness	0.29	0.67	0.34	0.61	0.83	0.643	0.7	0.334
Fullness	0.48	0.54	0.38	0.64	1.07	0.907	1	0.998
Nausea	0.3	0.69	0.36	0.62	0.94	0.957	0.83	0.65
Vomiting	0.17	0.94	0.61	0.65	2.95	0.024	2.81	0.052
Halitosis	0.25	0.73	0.36	0.62	0.91	0.885	0.92	0.836
Bile taste	0.29	0.7	0.36	0.62	0.95	0.984	0.91	0.816
Smoking	0.11	0.94	0.5	0.64	1.77	0.36	1.25	0.682
Alcohol	0.28	0.83	0.49	0.66	1.85	0.089	1.47	0.336

PPV: Positive predictive value; NPV: Negative predictive value; SI: Symptom index.

receiver operating characteristics (ROC) of the respective symptoms in association with PPI response were calculated (Figure 1).

DISCUSSION

The aim of this study was to identify objective and subjective predictors for the reliable diagnosis of GERD and the reported therapeutic response to PPIs to facilitate a more focused therapeutic approach in the future. Predicting the success of PPI therapy in symptomatic patients suspected of GERD would be helpful for preventing futile trials of empiric PPI medication and repeated reflux measurements and for reducing health care costs. In particular, the therapeutic response rates in patients with non-erosive reflux disease (NERD) and atypical/extra-esophageal symptoms are not satisfactory[17]. To solve this problem, an effort was made to discriminate NERD from functional disorders (FD) with special pH/MII patterns. However, despite the known overlap between FD and reflux symptoms, approximately 38% of FD patients also report symptom relief upon PPI therapy[12]. Therefore, the ultimate clinical implication is to specifically detect patients responding to PPIs, regardless of NERD, atypical/extraesophageal symptoms or FD.

The focus was based on patient characteristics and anamnestic parameters (gender, BMI, smoking habits, alcohol). As expected, patients with both typical reflux symptoms, such as heartburn, regurgitation or positive SI, and fullness and hoarseness sufficiently respond to PPIs. These anamnestic parameters are good predictors for PPI therapeutic success. According to our data, empirical PPI trials are warrantable in patients with the respective anamnestic data. Male gender, obesity and alcohol consumption are also associated with positive therapeutic responses to PPIs, which might be of high interest in the primary care setting. Interestingly, smoking habits were not significant predictors for the PPI response. Patients with objective pathological results from pH/MII also respond to PPIs sufficiently.

In nonspecific anamnesis, the pH/MII and the SI are comparable options for guiding PPI therapy. As shown in previous trials, pH/MII can potentially facilitate a more tailored therapeutic approach in patients with PPI-resistant GERD symptoms and ensures the success of further escalating PPI therapy[23]. In this retrospective analysis, we used the same objective parameters because the number of reflux episodes can be easily assessed in

Table 3 Parameters associated with a pathological symptom index

Parameters	Sensitivity	Specificity	PPC	NPV	OR (univ)	*P*val (univ)	OR (mult)	*P*val (mult)
Group A								
pH-metry	0.39	0.81	0.61	0.63	2.67	< 0.001	2.04	0.004
Gender	0.54	0.5	0.46	0.58	1.18	0.44	1.1	0.681
Bmi	0.49	0.6	0.49	0.6	1.42	0.077	1.14	0.547
Heartburn	0.7	0.52	0.54	0.69	2.6	< 0.001	1.28	0.34
Regurgitation	0.7	0.6	0.58	0.72	3.42	< 0.001	2.73	< 0.001
Globus sensation	0.49	0.59	0.48	0.6	1.4	0.092	1.35	0.174
Burning tongue	0.22	0.77	0.43	0.56	0.97	0.97	0.66	0.113
Coughing	0.47	0.6	0.48	0.59	1.36	0.129	1.18	0.49
Hoarseness	0.43	0.66	0.5	0.6	1.46	0.062	1.15	0.553
Fullness	0.58	0.58	0.52	0.64	1.91	0.001	1.34	0.206
Nausea	0.34	0.8	0.57	0.61	2.11	0.001	1.13	0.665
Vomiting	0.12	0.93	0.59	0.58	2	0.048	1.56	0.252
Halitosis	0.26	0.74	0.44	0.56	1.03	0.958	0.65	0.085
Bile taste	0.29	0.78	0.5	0.58	1.42	0.129	1.01	0.952
Imp path	0.34	0.84	0.63	0.62	2.77	< 0.001	2.26	0.002
Smoking	0.07	0.93	0.44	0.56	1.01	1	1.1	0.808
Alcohol	0.22	0.74	0.4	0.55	0.81	0.402	0.85	0.537
Group B								
pH-metry	0.33	0.91	0.7	0.68	5.03	< 0.001	3.28	0.006
Gender	0.49	0.47	0.37	0.6	0.86	0.687	0.86	0.678
BMI	0.42	0.54	0.36	0.6	0.85	0.642	0.59	0.115
Heartburn	0.66	0.41	0.41	0.66	1.36	0.353	1.32	0.492
Regurgitation	0.65	0.44	0.42	0.67	1.5	0.198	1.19	0.664
Globus sensation	0.43	0.6	0.4	0.63	1.13	0.776	0.84	0.62
Burning tongue	0.21	0.77	0.37	0.61	0.9	0.895	1.08	0.841
Coughing	0.5	0.61	0.45	0.66	1.58	0.128	1.56	0.22
Hoarseness	0.36	0.71	0.44	0.64	1.42	0.299	1.34	0.43
Fullness	0.51	0.55	0.42	0.64	1.3	0.407	1.21	0.588
Nausea	0.33	0.7	0.41	0.62	1.13	0.791	1	0.995
Vomiting	0.15	0.93	0.56	0.63	2.26	0.101	1.67	0.347
Halitosis	0.27	0.74	0.39	0.62	1.02	1	1.05	0.907
Bile taste	0.3	0.71	0.39	0.62	1.05	0.989	0.84	0.653
Imp path	0.5	0.84	0.66	0.73	5.23	< 0.001	4.84	< 0.001
Smoking	0.1	0.93	0.5	0.62	1.66	0.431	2.06	0.21
Alcohol	0.23	0.8	0.43	0.62	1.24	0.643	1.03	0.941

PPV: Positive predictive value; NPV: Negative predictive value; pH/MII: pH-metry/impedance measurement.

a standardized manner and because reference values are available. As a subjective parameter, the SI was evaluated. All parameters used in the pH/MII were able to predict the PPI response with a comparable odds ratio to that of typical GERD symptoms. Because of the strong correlation of pH/MII with GERD, we analyzed the anamnestic parameters associated with pathological parameters in pH/MII. Again, a positive SI and regurgitation were associated, but there were also positive associations with increased BMI and male gender. Analyzing the SI did not reveal any new aspects.

Hence, an index empiric PPI trial is a warrantable option in patients with typical reflux symptoms (heartburn and regurgitation), male gender, obesity or atypical GERD symptoms (fullness, hoarseness). Furthermore, in accordance with our data, pH/MII is a reliable tool for guiding therapy if the anamnesis is inconclusive. Anamnestic parameters, including gender, obesity and drinking habits, also predict therapy response.

More conflicting are our results in patients who were assessed while their PPI therapies were ongoing. The indications for pH/MII were persistent symptoms despite PPI or therapy monitoring. Neither anamnestic nor pH/MII parameters were evaluable for predicting the PPI response. One might argue that the number of reflux episodes of 73 fluid and/or mixed reflux episodes within 22-24 h used in this study is too high because these values were generated in patients who were off PPI therapy. However, we also analyzed the SI as a subjective parameter. Nonetheless, it was not possible to predict the PPI response. On the one hand, it may therefore be assumed that the number of FD patients is most likely higher in the "non-responding group". This assumption is supported by the high number of normal pH/MII results in the "on therapy" group. On the other hand, it is known that patients who are unresponsive to standard PPI therapy respond to escalating PPI therapy in up to 90% of cases[23]. However, the effect of an escalating PPI dose was not analyzed in this trial. In clinical practice, patients with persistent symptoms despite standard therapy should undergo pH/MII testing. If the results are pathological, then escalating PPI therapy is a promising treatment[23]. In PPI-unresponsive patients, extra-esophageal signs and symptoms are more likely due to causes other

than GERD. Continued PPI therapy in this group is not recommended[24].

From previous reports and in accordance with our data, increased BMI is a risk factor for GERD[23], and pH/MII monitoring reveals pathologic findings particularly in these patients. It is also known that persistent gastroesophageal reflux despite standard PPI-therapy is a common problem in patients with increased BMIs (> 25). Therefore, a possible explanation is increased intra-abdominal pressure due to adipose tissue, leading to increased gastric pressure, decreased gastric emptying and consecutive relaxation of the lower esophageal sphincter[25,26]. In accordance with previous trials, we detected good clinical responses to standard PPI therapy in obese patients[27].

To the best of our knowledge, the present study provides the largest series of pH/MII data. The limitation of our study is its single-center setting. Furthermore, it is noteworthy that certain methodological problems existed due to the retrospective approach. First, the patients were not subject to a previously created study protocol that involved the use of different PPI agents. Second, it was not possible to precisely monitor PPI intake prior to the pH/MII. Notwithstanding, we believe that the high number of patients suffices as a robust database.

In conclusion, in patients who are off PPIs and have typical reflux symptoms (heartburn and regurgitation), male gender, obesity or atypical GERD symptoms (fullness, hoarseness), empiric PPI therapy is most likely to be successful. In non-obese, female patients with non-typical reflux symptoms, pH/MII (including evaluation of the SI) should be considered instead of empiric PPI therapy. Anamnestic parameters, including gender, obesity and drinking habits, also predict the therapeutic response. With respect to predicting the therapeutic response, pH/MII during ongoing PPI therapy is not useful. Thus, particularly in primary care settings, a more focused therapeutic approach should be conducted instead of treating patients empirically, thereby avoiding ineffective, long-term PPI trials in the future.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is one of the most prevalent gastrointestinal disorders. The general therapeutic aim is the relief of symptoms and the prevention of associated complications. Standard empiric proton pump inhibitor (PPI) therapy poses a substantial economic burden and yields varying success.

Research frontiers

pH/impedance monitoring (pH/MII) provides a reliable pattern for discriminating reflux disease and functional disorders. Reliable objective parameters or specific anamnestic findings for predicting the therapeutic response to PPIs in high-volume studies are absent.

Innovations and breakthroughs

The present study provides the largest series of pH/MII data to identify objective and subjective predictors for the reliable diagnosis of GERD and the response to PPI therapy.

Applications

In patients off PPIs and with typical reflux symptoms, male gender, obesity or atypical GERD symptoms, empiric PPI therapy is most likely to be successful. In non-obese, female patients with non-typical reflux symptoms, pH/MII should be applied instead of empiric PPI therapy. With respect to predicting the therapeutic response, pH/MII during ongoing PPI therapy is not useful.

Terminology

Up to 40% of the adult population suffers occasionally from reflux symptoms. Symptoms are subdivided into typical (heartburn, regurgitation) and atypical symptoms (chronic cough, hoarseness, recurrent sinusitis, globus sensations in the throat, a burning feeling on the tongue, dental erosions, fullness). The most common therapeutic approaches are PPI trials, which have variable success.

Peer review

This manuscript provides systematic theoretical analyses and valuable conclusions.

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RESEARCH REPORT

Non-small-bowel abnormalities identified during small bowel capsule endoscopy

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Abstract

AIM: To investigate the incidence of non-small-bowel abnormalities in patients referred for small bowel capsule endoscopy, this single center study was performed.

METHODS: Small bowel capsule endoscopy is an accepted technique to investigate obscure gastrointestinal bleeding. This is defined as bleeding from the digestive tract that persists or recurs without an obvious etiology after a normal gastroduodenoscopy and colonoscopy. Nevertheless, capsule endoscopy sometimes reveals findings outside the small bowel, *i.e.*, within reach of conventional endoscopes. In this retrospective single center study, 595 patients undergoing capsule endoscopy between 2003 and 2009 were studied. The incidence of non-small bowel abnormalities was defined as visible abnormalities detected by capsule endoscopy that are located within reach of conventional endoscopes.

RESULTS: In 595 patients, referred for obscure gastrointestinal bleeding or for suspected Crohn's disease, abnormalities were found in 306 (51.4%). Of these 306 patients, 85 (27.7%) had abnormalities within reach of conventional endoscopes; 63 had abnormalities apparently overlooked at previous conventional endoscopies, 10 patients had not undergone upper and lower endoscopy prior to capsule endoscopy and 12 had abnormalities that were already known prior to capsule endoscopy. The most common type of missed lesions were vascular lesions (n = 47). Non-small-bowel abnormalities were located in the stomach (n = 15), proximal small bowel (n = 22), terminal ileum (n = 21), colon (n = 19) or at other or multiple locations (n = 8). Ten patients with abnormal findings in the terminal ileum had not undergone examination of the ileum during colonoscopy.

CONCLUSION: A significant proportion of patients undergoing small bowel capsule endoscopy had lesions within reach of conventional endoscopes, indicating that capsule endoscopy was unnecessarily performed.

Key words: Capsule endoscopy; Small bowel; Findings; Colon; Stomach

Core tip: Capsule endoscopy (CE) is a technique to detect small bowel pathology which sometimes reveals non-small bowel abnormalities (NSBAs). There are no data on the incidence of NSBAs in capsule endoscopy. In this study, 595 capsule endoscopy procedures were included. Abnormalities were found in 306 (51.4%) of cases. Of these 306 patients, 85 (27.7%) had abnormalities within reach of conventional endoscopes. The fact that a significant proportion of patients referred for small bowel CE had lesions within the reach of conventional endoscopes indicates that CE was unnecessarily performed and emphasizes the importance of critical selection of patients for capsule endoscopy.

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INTRODUCTION

Small bowel capsule endoscopy (CE) has become an established method for visualization of the small bowel[1-4]. One of the main indications for CE is obscure gastrointestinal bleeding. Obscure gastrointestinal bleeding is defined as bleeding from the digestive tract that persists or recurs without an obvious etiology after a normal esophagogastroduodenoscopy and colonoscopy[1]. It can be categorized into overt and occult obscure gastrointestinal bleeding based on the presence or absence of clinically evident bleeding. Approximately 5% of patients presenting with gastrointestinal bleeding have no identified source on upper endoscopy and colonoscopy[1]. The cause of obscure gastrointestinal bleeding is usually a lesion located in the small bowel, but also includes lesions that were overlooked during conventional endoscopy, either because of intermittent bleeding or truly missed lesions. Another important indication for small bowel capsule endoscopy is suspected Crohn's disease (CD). Usually, prior to the procedure, colonoscopy is being performed, preferably including endoscopic inspection of the terminal ileum. So, most patients referred for small bowel capsule endoscopy have undergone conventional endoscopies prior to the procedure.

Another method to investigate the small bowel is single- or double balloon enteroscopy[5,6]. As in capsule endoscopy, most patients referred for balloon enteroscopy have undergone conventional upper and lower endoscopy before the procedure is conducted[7]. It is known that balloon enteroscopy reveals abnormalities within reach of conventional endoscopes in up to 15%-24% of patients[8,9]. These findings are generally referred to as non-small-bowel abnormalities (NSBAs)[8-10]. There are no firm data on the incidence of NSBAs in capsule endoscopy. The aim of this study was therefore to determine the incidence of findings within reach of conventional endoscopes in patients referred for CE.

MATERIALS AND METHODS

Patients and techniques

Data from all consecutive CE studies performed at the University Medical Centre Groningen, the Netherlands, between September 2003 and January 2009 were prospectively collected. Our hospital is a tertiary-care centre with a referral base drawing from the northern part of the Netherlands. Data were collected on patient demographics, indications for the procedure, procedural data, including gastric and small bowel transit time, and findings of the procedure. CE was considered complete when the cecum was reached within recording time. NSBAs were defined as all abnormal findings found at capsule endoscopy located in the stomach, proximal small bowel, terminal ileum, and colon. Data were retrieved with respect to the extent and number of endoscopies performed prior to CE and whether or not ileoscopy was performed during colonoscopy. In case of missing data from externally referred patients, the referring hospital was contacted or visited.

CE procedure

All patients received the same bowel preparation during the study period. The patients were given standardized instructions before the procedure, and informed consent was obtained. The patients were asked to stop iron supplements seven days before CE and to use a low-fiber diet 3 d before CE. The patients started a fasting period at midnight before the procedure. Bowel preparation consisted of four liters of polyethylene glycol (PEG), given as 3 L the evening before the procedure and 1 L in the morning. The capsule (Pillcam; Given Imaging Ltd, Yoqneam, Israel) was swallowed in the morning. The patients were allowed to drink fluids after 3 h and to consume a light meal after 5 h. Before capsule ingestion, 100 mL of antifoam and a prokinetic agent was given, 10 mg of domperidone (before July 1st 2008, n = 641) or 250 mg of erythromycin (after July 1st 2008, n = 69). All CE procedures were reviewed by two gastroenterologists, experienced with capsule endoscopy (Weersma R and Koornstra JJ). Controversial findings were discussed, and consensus was reached upon the final diagnosis. The most relevant findings obtained from CE were documented and categorized according to standard terminology (10) as angiectasia(s); ulcer(s); active bleeding of unknown origin; erosion(s); polyp(s)/tumor(s); incidental abnormality of esophagus, stomach, or colon; no abnormality; or unable to make a diagnosis.

Statistical analysis

P values below 0.05 were considered significant. SPSS 14.0 for Windows software (SPSS Inc., Chicago, IL, United States) were used for statistical analyses.

RESULTS

During the study period, 710 capsule endoscopy procedures were performed in 674 patients. 389 patients were female (54.8%) and the average age was 55 years (range 9-93, SD 18). Most of the patients were referred for capsule endoscopy for analysis of obscure-occult gastrointestinal bleeding (n = 392, 55.2%), obscure-overt gastrointestinal bleeding (n = 87, 12.3%) or suspected CD (n = 116, 16.3%). Given the aim of our study, further analysis was limited to these 595 patients. 331 patients (55.6%) were referred by physicians from other hospitals.

Table 1 Findings of capsule endoscopy procedures (595 procedures) *n* (%)

Procedures	*n* = 595
No abnormalities	289 (48.6)
Angiodysplasia(s)	115 (19.3)
Erosion(s)	68 (11.4)
Ulcer(s)	34 (5.7)
Polyp/tumor	31 (5.2)
Active bleeding	28 (4.7)
Other	30 (5.0)

Previous examinations and capsule endoscopy findings

Patients had undergone a mean number of 1.1 (range 0-5) esophagogastroduodenoscopy procedures and 1.1 (range 0-9) colonoscopy procedures prior to capsule endoscopy. During colonoscopy, the terminal ileum had been intubated in 41.2% of patients. In addition to conventional endoscopy procedures, 20.6% of patients had undergone a small-bowel-follow-through examination and 9.9% of patients had undergone a push-enteroscopy prior to CE. The coecum was reached within recording time in 487 (81.8%) of capsule endoscopy procedures. Findings of capsule endoscopy are summarized in Table 1. In 291 CE procedures, abnormalities were found. The most common abnormal findings were angiodysplasias (n = 115, 19.3%) and erosion(s) (n = 68, 11.4%).

Non-small-bowel abnormalities

In 85 patients (14.3%), abnormalities were found within reach of conventional endoscopes, summarized in Table 2. In most patients (n = 63, 10.6%), this concerned unknown abnormalities in patients that had undergone both gastroduodenoscopy and colonoscopy prior to CE. In 10 patients (1.7%), NSBAs were found while patients had not undergone esophagogastroduodenoscopy and ileocolonoscopy prior to CE and in 12 patients (2.0%), NSBAs were found that were already known prior to capsule endoscopy. NSBAs were located in: stomach (n = 15), duodenum (n = 12), proximal jejunum (n = 10), terminal ileum (n = 21), colon (n = 19) or at other or at multiple locations (n = 8). The types of lesions encountered are summarized in Table 2: angiodysplasias (n = 32, 37.6%), erosion(s) (n = 16, 18.8%), active bleeding (n = 15, 17.6%) and inflammatory lesions (n = 12, 14.1%). 59 of 85 patients (69.4%) with NSBAs concerned patients referred from other hospitals. CD was suspected in 116 of 595 patients (19%). Abnormalities in the terminal ileum were seen in 39 patients (33.6%). In only 12 of these 39 patients (30.8%), the terminal ileum had been inspected during previous colonoscopy.

Table 2 Non-small-bowel abnormalities in capsule endoscopy *n* (%)

Procedures	Value
Abnormalities	291 (48.9)
NSBA	85 (14.3)
NSBA known before CE	12 (2.0)
NSBA unknown before CE	63 (10.6)
sNSBA with incomplete previous examinations	10 (1.7)
Location of NSBA	
Stomach	15 (17.6)
Duodenum	12 (14.1)
Proximal jejunum	10 (11.8)
Terminal ileum	21 (24.7)
Colon	19 (22.4)
Other	8 (9.4)
Type of NSBA	
Angiodysplasia(s)	32 (37.6)
Erosion(s)	16 (18.8)
Active bleeding	15 (17.6)
Inflammation	12 (14.1)
Polyp/tumor	6 (7.1)
Other	4 (4.7)

NSBA: Non-small bowel abnormalitie; CE: Capsule endoscopy.

DISCUSSION

In this study, we found that in patients referred for capsule endoscopy it is not uncommon to find non-small-bowel abnormalities, so findings within the reach of conventional esophagogastroduodenoscopy or ileocolonoscopy related to the indication for the procedure. We included only patients who were referred for obscure or occult bleeding and for suspected CD, because patients with other indications for CE, such as suspicion of carcinoid do not generally undergo both esophagogastroduodenoscopy and ileocolonoscopy prior to CE.

Non-small-bowel abnormalities within reach of conventional endoscopes were found in 14.3% of all procedures and could be present in the upper and lower gastrointestinal tract. Vascular lesions were the abnormalities most often found. It must be noted that two-thirds of these patients had undergone conventional upper and lower tract endoscopy with ileoscopy before the capsule endoscopy procedure. One could assume that these lesions were truly overlooked at previous examinations. Alternatively, it may concern intermittently bleeding lesions.

Data on the incidence of non small bowel abnormalities in CE are limited. To the best of our knowledge, only two studies investigated this subject. In a series of 140 capsule endoscopy procedures for obscure gastrointestinal bleeding, NSBA were found in 9 patients (6.4%)[7]. In another series of 317 CE procedures, NSBA were found in 11 patients (3.5%), in which the investigators differentiated between referred patients (6.3%) and non-referred patients (1.2%). In this study, the terminal ileum was not defined as a location for NSBA[11]. The incidence of NSBA has also been investigated in double balloon endoscopy (DBE) procedures[8,9]. In these studies, NSBAs were found in 14.3 % and 24 % of cases respectively. One could assume that the sensitivity of DBE is slightly higher for small bowel abnormalities than that of CE, although most studies indicate a similar diagnostic yield[5,12-21].

In this study we investigated the incidence of NSBAs in relation to prior examinations. A limitation of our

study is that we do not have follow-up data on the patients in whom NSBAs were found. We therefore are not informed of the results of repeat conventional endoscopies after CE. A strong point of our patient cohort is that by selecting only patients who were referred for obscure of occult blood loss and for suspected CD, a group of patients generally fully examined with conventional endoscopies prior to CE.

In conclusion, a significant proportion of patients referred for small bowel CE had lesions within the reach of conventional endoscopes, indicating that CE was unnecessarily performed. Before planning a CE procedure, careful upper and lower endoscopies should be performed including ileoscopy. Repeating these investigations, if not properly performed before CE, should be considered.

COMMENTS

Background

Capsule endoscopy (CE) is a very sensitive diagnostic technique to detect small bowel pathology. Another method to investigate the small bowel is single- or double-balloon enteroscopy. As in capsule endoscopy, most patients referred for balloon enteroscopy have undergone conventional upper and lower endoscopy before the procedure is conducted. It is known that balloon enteroscopy reveals abnormalities within reach of conventional endoscopes in up to 15%-24% of patients. These findings are generally referred to as non-small-bowel abnormalities. There are no robust data on the incidence of NSBAs in capsule endoscopy. This was the subject of this study.

Research frontiers

This the first study that investigated the incidence of non-small-bowel abnormalities in small bowel capsule endoscopy.

Innovations and breakthroughs

In this study, 595 capsule endoscopy procedures were included. Patients were referred for obscure gastrointestinal bleeding or for suspected Crohn's disease. Abnormalities were found in 306 (51.4%) of cases. Of these 306 patients, 85 (27.7%) had abnormalities within reach of conventional endoscopes; 63 had abnormalities apparently overlooked at previous conventional endoscopies, 10 patients had not undergone upper and lower endoscopy prior to capsule endoscopy and 12 had abnormalities that were already known prior to capsule endoscopy. The most common type of missed lesions were vascular lesions (n = 47). Non-small-bowel abnormalities were located in the stomach (n = 15), proximal small bowel (n = 22), terminal ileum (n = 21), colon (n = 19) or at other or multiple locations (n = 8). Ten patients with abnormal findings in the terminal ileum had not undergone examination of the ileum during colonoscopy.

Applications

What does this mean for clinical practice? The fact that a significant proportion of patients referred for small bowel CE had lesions within the reach of conventional endoscopes indicates that CE was unnecessarily performed. Before planning a CE procedure, careful upper and lower endoscopies should be performed including ileoscopy. Repeating these investigations, if not properly performed before CE, should be considered.

Peer review

This work emphasizes the importance of critical selection of patients for capsule endoscopy.

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RESEARCH REPORT

Combined laparoscopic spleen-preserving distal pancreatectomy and islet autotransplantation for benign pancreatic neoplasm

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Abstract

AIM: To evaluate the safety and feasibility of laparoscopic spleen-preserving distal pancreatectomy (LSPDP) with autologous islet transplantation (AIT) for benign tumors of the pancreatic body-neck.

METHODS: Three non-diabetic, female patients (age 37, 44 and 35 years, respectively) were declared candidates for surgery, between May and September 2011, because of pancreatic body/neck cystic lesions. The planned operation was an LSPDP associated with AIT from the normal pancreas distal to the neoplasm. Islets isolation was performed on the residual pancreatic parenchyma after frozen section examination of the margin. Purified autologous islets were infused into the portal vein by a percutaneous transhepatic approach the day after surgery.

RESULTS: The procedure was performed successfully in all the three cases, and the spleen was preserved along with its vessels. Mean operation time was 283 ± 52 min and average blood loss was 133 ± 57 mL. Residual pancreas weights were 33, 22 and 30 g, and 105.200, 40.390 and 94.790 islet equivalents were isolated, respectively. Surgical complications occurred in one patient (grade A pancreatic fistula). Postoperative stays were 6, 6 and 7 d, respectively. Histopathological evaluation revealed mucinous cystic neoplasm in cases 1 and 3, and serous cystic neoplasm in patient 2. No postoperative insulin administration was required. One patient developed a transient partial portal thrombosis 2 mo after islet infusion. Patients are insulin independent at a mean follow up of 8 ± 2 mo.

CONCLUSION: Combination of LSPDP and AIT is feasible and could be effective to minimize the surgical impact for benign neoplasm of pancreatic body-neck.

Key words: Pancreas; Benign neoplasm; Laparoscopy; Minimally invasive treatment; Spleen preservation; Pancreatogenic diabetes; Autologous islet transplantation

Core tip: The article describes, for the first time, a combination of techniques to reduce all possible consequences of pancreatic resection for benign/borderline neoplasms located at the pancreatic body-neck. The procedure combines laparoscopy, spleen preservation and islet autotransplantation. The laparoscopic approach reduces the access trauma of an extensive surgery. The spleen preservation avoids infectious and hematological complications related to splenectomy. Islet autotransplantation could reduce the incidence of pancreatogenic diabetes after resection.

Balzano G, Carvello M, Piemonti L, Nano R, Ariotti R, Mercalli A, Melzi R, Maffi P, Braga M, Staudacher C. Combined laparoscopic spleen-preserving distal pancreatectomy and islet autotransplantation for benign pancreatic neoplasm. *World J Gastroenterol* 2014; 20(14): 4030-4036 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4030.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4030

INTRODUCTION

Distal pancreatectomy (DP) is the standard operation for borderline pancreatic tumors located in the body-neck of the pancreas[1,2]. Spleen preservation is indicated in such cases to reduce immunological and hematological impairment[3].

Although there are no prospective randomized studies, there is agreement regarding the feasibility and efficacy of the laparoscopic approach compared with the open procedure[3-10].

DP is associated with the risk of post-surgical diabetes, ranging from 5% to 42%, related with the amount of resected parenchyma[11-15]. Autologous islet transplantation (AIT) is effective in the prevention of post-surgical diabetes, by rescuing the endocrine component of the non-neoplastic resected pancreas[16,17].

Minimizing the surgical damage is a challenging goal, especially in patients with a long life expectancy. In the setting of a benign neoplasm of the pancreatic body-neck, this goal could be achieved completely by the combination of a laparoscopic technique, spleen preservation and AIT; such an approach has not been reported yet. In this report, we describe three patients affected by pancreatic cystic neoplasm of the body-neck, successfully treated by laparoscopic spleen-preserving distal pancreatectomy (LSPDP) and AIT.

MATERIALS AND METHODS

Patient 1

A 37-year-old woman underwent a computerized tomography (CT) scan because of persistent postprandial abdominal pain. The CT-scan revealed a 4 cm cystic lesion in the body of the pancreas, suspected as being a mucinous cistadenoma (Figure 1A). Plasma carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9 levels were within the normal range. Fine needle biopsy, performed by endoscopic ultrasound, detected the presence of mucinous fluid associated with epithelial cells but without signs of dysplasia.

Patient 2

A 44-year-old woman presented with an abdominal epigastric mass associated with nausea and mild pain since 2 mo. A CT-scan showed a 10 cm cystic lesion of the pancreatic body (Figure 1B). There were no mural nodules or contrast-enhancement of the cyst wall. Plasma CEA and CA 19-9 values were normal.

Patient 3

A 35-year-old woman underwent a CT-scan because of left-sided renal colic. As a collateral finding, a pancreatic cystic lesion was described. Abdominal magnetic resonance imaging (MRI) showed a 5 cm unilocular cyst of the pancreatic neck suspected as being a mucinous cistadenoma, with a 1.5 cm intramural nodule (Figure 1C). Plasma CEA level was normal, while the CA 19-9 was elevated (421 UI/mL). A careful evaluation of the MR images showed no involvement of the surrounding pancreatic parenchyma, with a well-demarcated capsule of the cystic lesion and no dilation of the main pancreatic duct.

Surgical technique

The patient is placed in a supine split legs position with the surgeon standing between the legs. Four trocars are used. After establishing laparoscopic access, the gastrocolic omentum is divided, accessing the lesser sac. In patient 2 (cyst diameter 10 cm) the cyst was emptied to allow adequate exposition of the splenic vessels. A retropancreatic tunnel is accomplished anterior to the superior mesenteric vein, exposing the splenic vein. The inferior border of the pancreatic body is mobilized from right to left. The splenic artery is identified close to its origin and freed from its pancreatic adhesions. The gland is transacted (about two centimeters to the right of the neoplasm) by a single application of a linear stapling device (ECHELON FLEX™ Powered ENDOPATH® Stapler). The splenic vessels are then skeletonized, proceeding towards the splenic hilum. The surgical specimen is extracted by endocatch through Pfannenstiel incision. The specimen is transected distal from the lesion with a 1 cm safety margin (Figure 2). A frozen section examination of this margin is performed. The spared pancreas is processed for islet isolation if the margin is negative.

The described surgical procedure was performed for all three patients.

Islets isolation

Islets isolation process is performed by an automated method, as previously described[18,19]. Briefly, the pancreatic duct is perfused by a collagenase solution. After parenchymal distension, the organ is digested and the islets are freed from the exocrine tissue. Using a COBE machine the islets are then purified.

Islets re-infusion

Under ultrasound guidance, using a percutaneous transhepatic approach, the islets are infused into the right portal vein.

Follow up of islets function and metabolic assessment

Capillary blood glucose was measured four times per day during the postoperative hospital stay. A mixed meal tolerance test was performed 1 month after transplantation. Fasting serum C-peptide, insulin and hemoglobin A1c

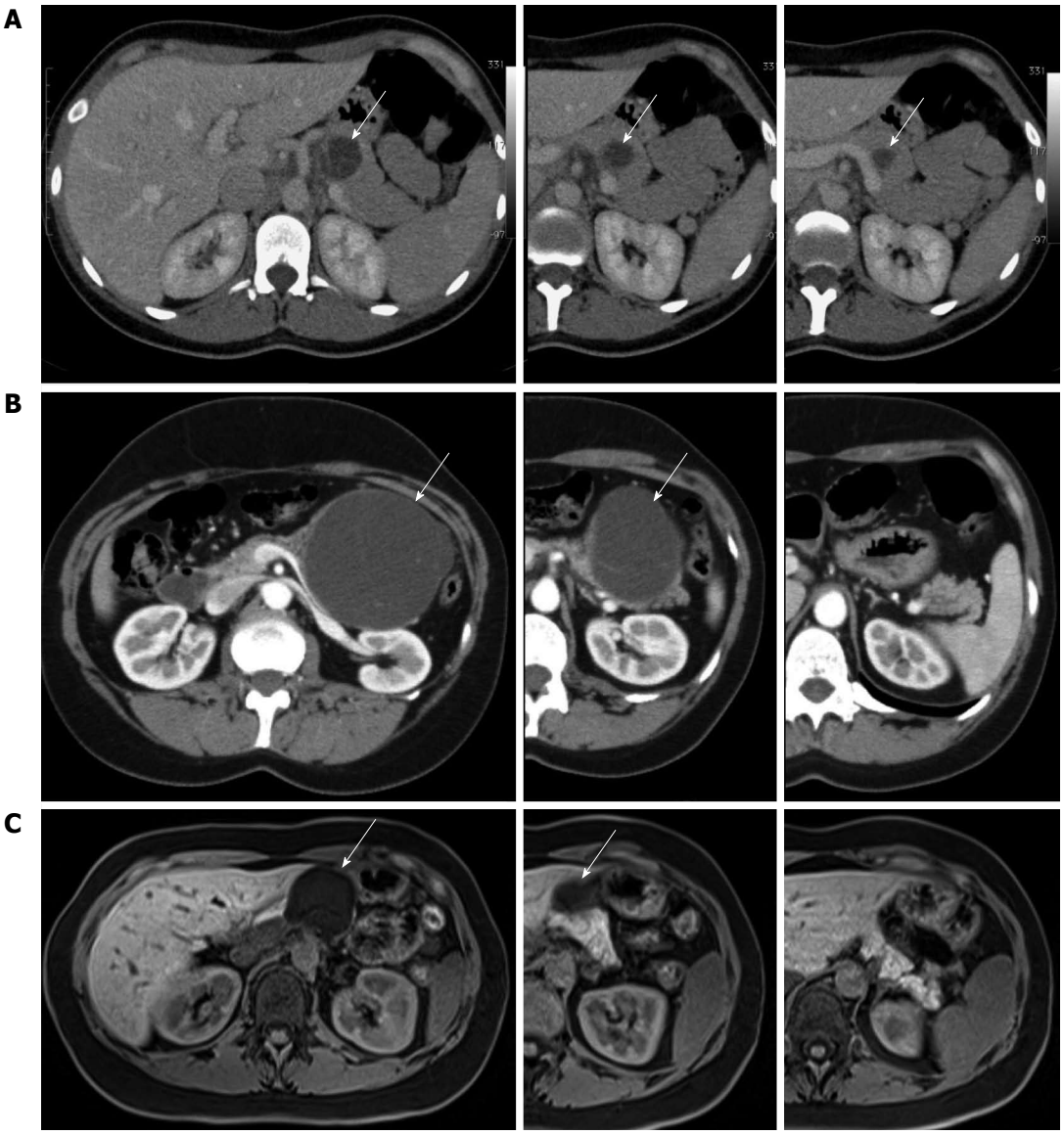

Figure 1 Abdominal computed tomography scan. A: Abdominal computed tomography (CT) scan showing a 4 cm pancreatic cystic lesion of the neck in patient 1; B: Abdominal CT scan showing a 10 cm pancreatic cystic lesion of the body in patient 2; C: Abdominal magnetic resonance imaging showing a 4.6 cm cystic lesion of the neck in patient 3. Arrows mark the cystic lesion (A-C).

were measured at 15 d and at 1, 2, 3 and 6 mo after transplantation.

RESULTS

Operations were performed laparoscopically, with no conversion; the spleen was preserved along with the splenic vessels. The average blood loss was 133 ± 57 mL. The mean operative time was 283 ± 52 min. Intraoperative frozen section examination of the pancreatic margin was normal. In patient 3, because of the cyst's characteristics and CA 19-9 elevation, an intraoperative frozen section examination of the cyst wall was performed to rule out malignancy. Pathological evaluation showed a mucinous cystoadenoma for patients 1 and 3, and a serous cystodenoma for patient 2. No major complication occurred during the operation or in the early postoperative period. Patient 3 developed a grade A pancreatic fistula (ISGPF definition[20]); the surgical drain was removed on postoperative day 36. Hospital stays were 6, 6 and 7 d, respectively.

The weights of the processed pancreatic parenchymas were 30, 22 and 33 g for patients 1-3, respectively. After the isolation process, 94.790, 40.390 and 105.200 islet equivalents (IEs) were obtained at the final count, while absolute islet numbers were 268.000, 95.000 and 274.800, respectively. IE per kilogram of body weight (IE/kg) were 1528, 553 and 1696, respectively. Portal vein pressure remained stable after islet infusion. No bleeding occurred after islet infusion, and regular portal system patency was documented the day after reinfusion by ultrasound assessment. No postoperative insulin administration was required. One month after surgery, patient 3 was diagnosed with partial portal vein thrombosis by

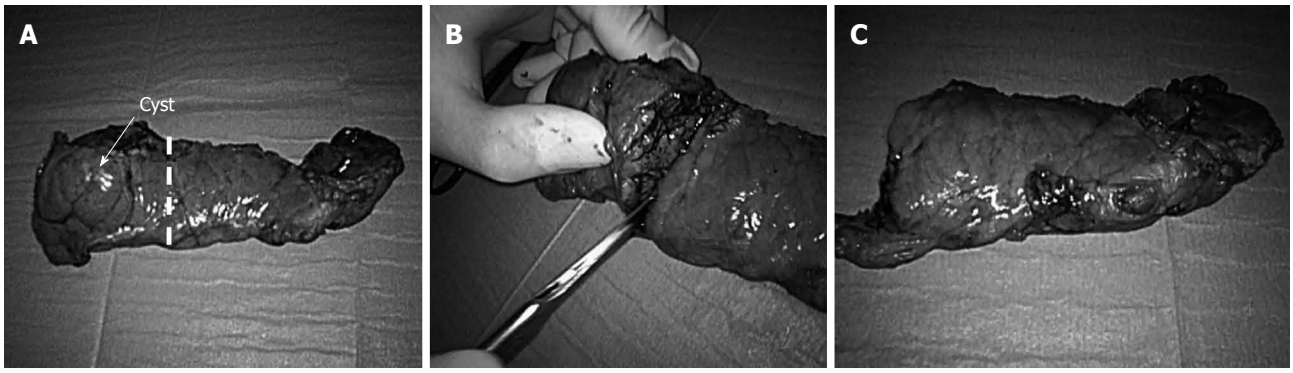

Figure 2 Specimen after distal pancreatic resection. A: An arrow marks the cystic lesion; the interrupted line marks the site of transection; B: Transection of the pancreas distal from the lesion; C: Spared pancreatic parenchyma.

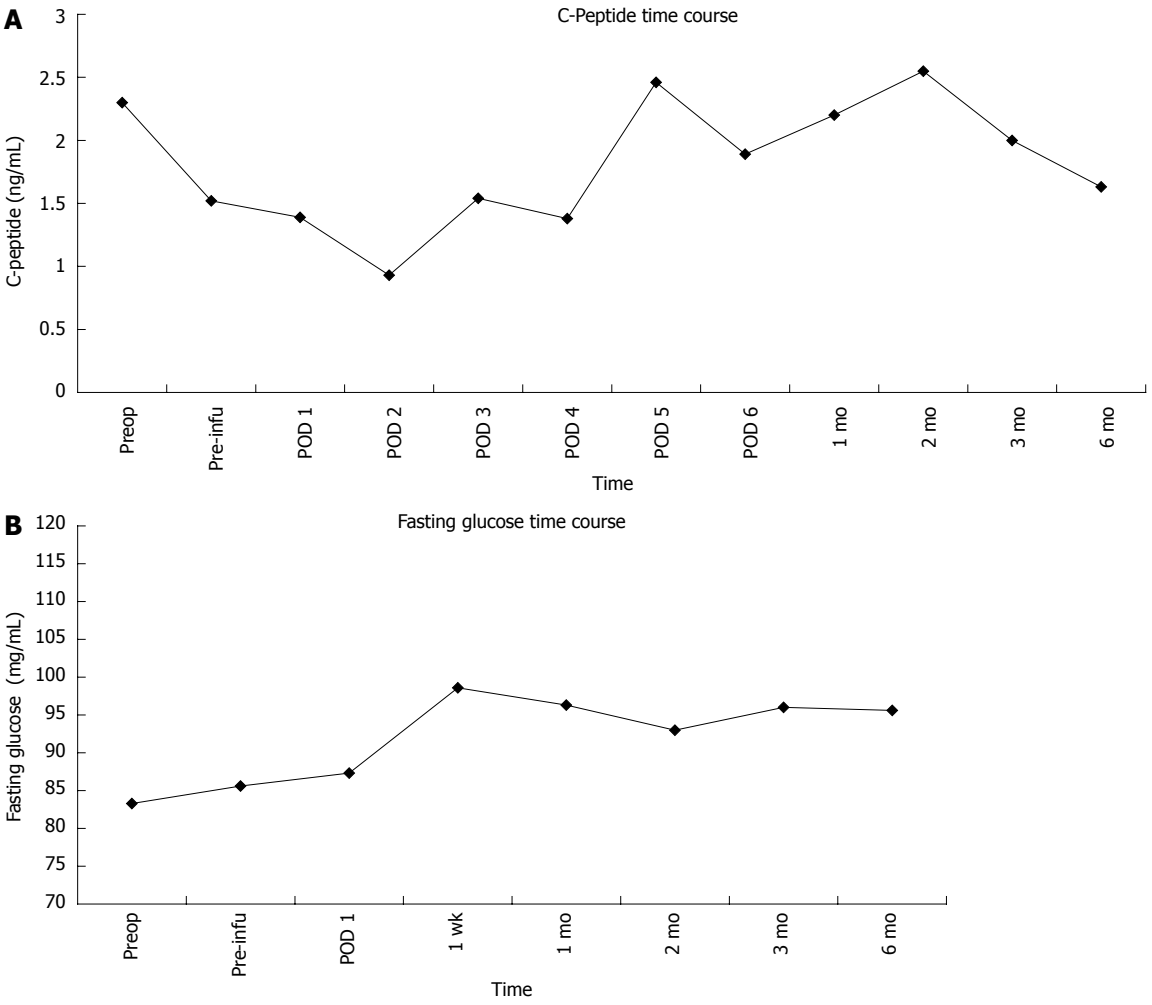

Figure 3 Fasting C-peptide (A) and fasting glycemia (B) remained stable and comparable with respect to pretransplant value, respectively. The values are expressed as mean plus the standard error of the mean. Preop: Preoperative value; Preinfu: C-peptide value after resection and before islet re-infusion; POD: Post operative day.

Doppler ultrasound, and was treated with low-molecular weight heparin. Metabolic assessment showed normal insulin production during follow-up. C-peptide values were comparable to preoperative values and remained stable during follow-up (Figure 3A). Fasting glucose remained stable after transplantation compared with the pre-transplant level (Figure 3B). The three patients were insulin independent at a median follow-up of 8 ± 2 mo.

DISCUSSION

Traditionally, DP is associated with splenectomy, and it is performed through a wide incision. For a borderline neoplasm of the body-neck, the removal of a significant

healthy portion of the gland is required, with consequent risk of pancreatogenic diabetes.

LSPDP with AIT may be a paradigm of the surgical evolution towards minimizing the operative short- and long-term impact. The mainstays of this procedure are the reduction of access trauma through laparoscopy, the avoidance of immunological and hematological long-term consequences of splenectomy, and the improvement of postoperative glycemic control by AIT. The advantages of laparoscopy have not yet been proved by any randomized trial comparing laparoscopic versus laparotomic DP. However, several studies showed the benefits of the laparoscopic approach, and no difference between the two procedures in terms of complications have been reported[4-6,21-23].

In our case series, no conversion was required, the mean surgical time was 266 ± 37 min, and, despite the AIT procedure, hospital stay was 6-7 d. Grade A fistula occurred in one of the three patients.

Splenectomy may result in long-term immunological and hematological impairment[24-26]. Spleen-preservation is therefore indicated for patients undergoing distal pancreatic resection for benign and low-grade malignant lesions[23]. Moreover, after spleen preservation, a reduction in early postoperative complications, especially infective morbidity, has been reported[27].

The incidence of new-onset diabetes after DP is probably underestimated: a retrospective study on 125 non-diabetic patients reported a minimal rate of postoperative diabetes (7.5% when excluding patients with chronic pancreatitis)[15]. Patients with chronic pancreatitis have an increased risk (up to 42%) of developing diabetes after distal pancreatic resection, because the endocrine function of the organ is already impaired[12]. However, analysis of diabetes onset in hemipancreatectomized living-donors for pancreas transplantation showed an unexpectedly high rate of glucose metabolism impairment: 25% of donors had overt diabetes or glucose intolerance[28] and 40% had abnormalities of glucose metabolism 3-10 years after donation[29].

To solve this problem, median pancreatectomy has been proposed as a surgical strategy for the treatment of benign neoplasms of the pancreatic body-neck[30-33]. However, with respect to DP, median pancreatectomy has a higher risk of pancreatic fistula (50%)[32,34].

Islet autotransplantation is an alternative to median pancreatectomy to preserve the endocrine function of distal pancreas, without increasing the fistula risk. Ris *et al*[17] performed open DP and AIT successfully in 25 patients within a 17-year period. At a median follow-up of 90 mo, all the patients were insulin independent[17].

In our case series, we isolated pancreatic islets from the resected pancreases and re-infused them, preserving endocrine function and maintaining euglycemia in the early and late post-operative period. The expected complications of the transhepatic islet infusion are low, mainly related to minor intra-and perihepatic bleeding and transient portal thrombosis[35,36]. One of three patients was diagnosed with partial intrahepatic thrombosis one month after islet infusion, which was successfully treated by low molecular weight heparin therapy.

When a conservative operation is planned, accurate consideration has to be made concerning the possibility of unsuspected malignancy. Indeed, the reported rate of malignancy in three large series of spleen-preserving DP published in 2012, was < 1% (two out of 213 overall patients)[23,37,38]. In case of suspicion of malignancy, endoscopic ultrasonography can provide a more accurate description of the lesion morphology, allowing fine needle aspiration (FNA) to be performed for cytological evaluation of the cyst fluid or of the solid component of the cyst wall. The dissemination risk of endoscopic FNA in presumed benign lesions was never reported, but even in cases of pancreatic malignancy, endoscopic FNA is considered not to increase the risk of peritoneal dissemination[39].

A further concern regards the possibility of occult malignancy in the normal pancreas segment to be processed for AIT. Pre- and intra-operative work up is essential to select adequate cases for autotransplantation. In our protocol, the presence of any multifocal pancreatic neoplasm at preoperative imaging or intraoperative evaluation, including multifocal benign intraductal-papillary mucinous neoplasm or a diagnosis (suspected or ascertained) of multiple endocrine neoplasm is an exclusion criterion. In the retrospective study carried out by Ris *et al*[17], 3 mm was considered a suitable safety margin, as demonstrated by the postoperative follow up. In the 90 mo median follow-up, all the patients were disease free. In our short series, pancreatic specimens were sent for frozen section analysis of the margin, with a margin of 1 cm. Furthermore, in patient 3, because of the preoperative findings, a frozen section examination of the cyst wall was performed to look for mural nodules and rule out malignancy before islet infusion.

The ultimate goal of this technique is to reduce the morbidity of extended pancreatic resection, required for patients with benign pancreatic tumor, occurring at the body-neck site. The minimally invasive treatment, with the preservation of the spleen and the vessels in association with AIT, may provide an improvement in early and late postoperative quality of life. However, while laparoscopic DP is increasingly performed, very few institutions may offer an islet producing facility. Besides the referral of patients to specific institutions, the creation of a network with neighboring hospitals should be considered to provide access to AIT to a wider number of candidate patients to this procedure[17].

To the best of our knowledge, this is the first study demonstrating the feasibility and safety of the minimally invasive spleen preserving left pancreatectomy combined with AIT. A larger patient series is needed to confirm the metabolic advantages of the technique.

COMMENTS

Background

Benign pancreatic lesion of the pancreatic neck might require extensive sur-

gery. The widespread use of laparoscopy has considerably reduced direct surgical trauma. Spleen preservation in such non-malignant cases has reduced the infectious morbidity of the standard procedure. However, with distal pancreatectomy, a considerable portion of the gland is removed, possibly leading to pancreatogenic diabetes. Islet auto transplantation is a promising strategy for reducing the risk of diabetes onset after pancreatic resection.

Research frontiers

This article could be important for the future evolution of minimally invasive procedures in pancreatic surgery.

Innovations and breakthroughs

Laparoscopy has already reduced the surgical trauma to patients. However, there may be lifelong metabolic consequences (pancreatogenic diabetes) as a result of pancreatic resection. Islet autotransplantation has been demonstrated as effective in the control of metabolic impairment after pancreatic resection. The main goal of this technique is to provide a combination of minimally invasive treatment and improvement of long term metabolic outcome.

Applications

The procedure has been demonstrated as feasible. A larger patient series is needed to confirm the metabolic advantages of the technique.

Terminology

Autologous islet transplantation is a procedure in which the endocrine component of the pancreas (islets of Langerhans) is preserved by a laboratory isolation process. The islets are then usually infused into the portal system by a percutaneous transhepatic approach.

Peer review

Interesting small series of autotransplantation in minimally invasive partial resection of benign pancreatic neoplasms.

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RESEARCH REPORT

Seasonal variations of acute appendicitis and nonspecific abdominal pain in Finland

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Abstract

AIM: To investigate whether seasonal changes had an effect on the incidence of acute appendicitis (AA) or nonspecific abdominal pain (NSAP).

METHODS: We carried out a national register study of all patients with a hospital discharge diagnosis of AA and acute NSAP in Finland. Data were analyzed for the whole country and correlated to seasonal and weather parameters (temperature, humidity). Moreover, additional sub-analyses were performed for five geographically different area of Finland.

RESULTS: The observation period spanned 21 years, with 186558 appendectomies, of which 137528 (74%) cases were reported as AA. The incidence of AA declined for 32% over the study period. The average incidence of the NSAP was 34/10000 per year. The mean annual temperature, but not relative humidity, showed clear geographical variations. The incidence of AA decreased significantly during the cold months of the year. No correlation was detected between temperature and incidence of NSAP. Humidity had a statistically significant impact on NSAP.

CONCLUSION: The incidence of acute appendicitis is declining in Finland. We detected a clear seasonality in the incidence of AA and NSAP.

Key words: Acute appendicitis; Appendectomy; Nonspecific abdominal pain; Incidence; Seasonal; Temperature; Humidity

Core tip: The incidence of acute appendicitis (AA) varies between countries and the etiology of the disease is still unclear. The aim of this study was to investigate whether seasonal changes had an effect on the incidence of AA or nonspecific abdominal pain (NSAP) in Finland between 1987 and 2007. We found that the incidence of AA, but not NSAP, was higher during the warm period of the year. In comparison to AA, NSAP was influenced by humidity and the incidence was lower during a period with lower levels of humidity.

Ilves I, Fagerström A, Herzig KH, Juvonen P, Miettinen P, Paajanen H. Seasonal variations of acute appendicitis and nonspecific abdominal pain in Finland. *World J Gastroenterol* 2014; 20(14): 4037-4042 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4037.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4037

INTRODUCTION

Acute appendicitis (AA) and nonspecific abdominal pain (NSAP) remain the two most common surgical emergen-

cies in acute care units[1-3]. NSAP has been evaluated in the literature as a self-limited diagnosis of acute abdominal pain for which no serious or definite cause is established and thus is no useful surgical treatment available[3]. Although AA has been recognized as a clinical entity for over 100 years[4], the differential diagnosis between AA and NSAP can still be challenging. Moreover, the etiology of the appendicitis still remains unclear and multifactorial. The disease has been attributed to a variety of possible causes, including mechanical obstruction[5], inadequate dietary fiber[6], smoking[7], air pollution[8] and familial susceptibility[9,10]. Population-based epidemiological studies about the impact of seasonal variables on appendicitis have been published, but so far there is no clear consensus about the connection between the seasonal variations and AA or NSAP[11,12].

The main factor influencing Finland's climate is the country's geographical position between the 60th and 70th northern parallels on the Eurasian continent, which shows characteristics of both maritime and continental climate. The mean temperature in Finland is several degrees (as much as 10 ℃ in winter) higher than that of other areas at these latitudes, *e.g.*, Siberia and south Greenland. The mean daily temperature can vary in Finland from below -30 ℃ during winter months (November-March) to more than +25 ℃ in the summer months (June-August). Moreover, because of the geographical shape and location of Finland there are large disparities in local temperatures inside the country. Finland has an excellent health register and is divided into five, geographically different University Hospital Districts (UHD) serving as a good base for comparing incidences and investigating various seasonal impacts on diseases. We have previously reported a declining incidence trend of AA, but not of NSAP[13]. The aim this study was to determine whether those trends could be explained by possible seasonal changes in temperature and humidity.

MATERIALS AND METHODS

A population-based register study was performed to assess the incidence of AA and NSAP in Finland between the years 1987 and 2007. Diagnoses were classified according to the World Health Organization International Classification of Diseases, version 9 and 10 (ICD-9 and ICD-10). The study population was recruited from the National Institute for Health and Welfare (NIHW) registry to retrieve the data on discharge diagnoses as well as surgical procedures of the whole country. Population data from 1987 to 2007 were retrieved from the Official Statistics of Finland. More detailed data about the material and methods can be found in our previously published study[13].

Weather data for the years 1987 to 2007 were retrieved from the Finnish Meteorological Institute[14] for each of the five UHD. Temperature and humidity levels were obtained from the main measurement points in each of the UHD, and were expressed as mean values for each month. Moreover, the temperature of the whole country was calculated as mean temperature value of each of the UHD. According to the measurement results, the period from March to September was defined as "warm period", and from October to February as "cold period".

Statistical analysis

Poisson regression analysis was used to assess association between temperature, humidity and incidences (AA and NSAP) for monthly data. In the Poisson regression model, incidences were as dependent variable per 10000 inhabitants. Temperature and humidity were explanatory variables. For the statistical analysis we used SPSS for Windows, release 18.0 (SPSS, Inc., Chicago, IL, United States).

RESULTS

During the study period, the mean annual temperature and relative humidity varied from 0.4 ℃-6.3 ℃ (mean 3.9 ℃) and 62%-76% (mean 69%), respectively (Figure 1). We observed cyclical temperature changes over a period of 4-5 years, but not in humidity. Lower levels of humidity were registered between 1996 and 2003. The mean temperature over the 21-year study period was below zero degrees from November to March (a cold period) (-4.1; SD 1.44 ℃), and above zero degrees in the warm period from April to October (10.6; SD 0.72 ℃) (Figure 2). Furthermore, a relative humidity showed higher values during the cold months. There was a large geographical disparity between temperatures of the different UHDs. The biggest difference between the mean temperatures was observed between the UHD of Oulu (Figure 3, area Ⅳ) 2.4 ℃ (min, -0.8 ℃; max, 4.5 ℃) and the UHD of Tampere (Figure 3, area Ⅲ) 5.0 ℃ (min, 1.6 ℃; max, 7.55 ℃), $P = 0.006$. During the study period, the annual temperature varied from +1.6 to +7.55 (median 1.7 ℃) and -0.75 to +4.5 (median 3.5 ℃) in the UHD of Tampere and Oulu, respectively, with no statistically significant difference in the incidence of AA between the hospital districts.

The incidence of AA declined for 32%, from 14.5 to 9.8 per 10000 inhabitants. The average incidence of NSAP was 34.0 per 10000 inhabitants. The incidence rose between the years 1987 and 1998 (from 25.2 to 39.8/10000 per year), after which it fell to 27.1/10000 per year. There was a clear difference in the incidence of AA between cold and warm months (Figures 2 and 4). The AA incidence was higher during the warm months throughout the study period. An increase of 10-Celsius degrees in temperature increased the incidence of AA by 4%, 1.04 (95%CI: 1.019-1.061). The incidence of NSAP did not change during the year, and the association between temperature and NSAP incidence was not statistically significant (Figure 4). Furthermore, humidity levels had no effect on incidence of AA, but had an effect on the incidence of NSAP. An increase of 10% in humidity decreased NSAP incidence by 0.8%, 0.992 (95%CI:

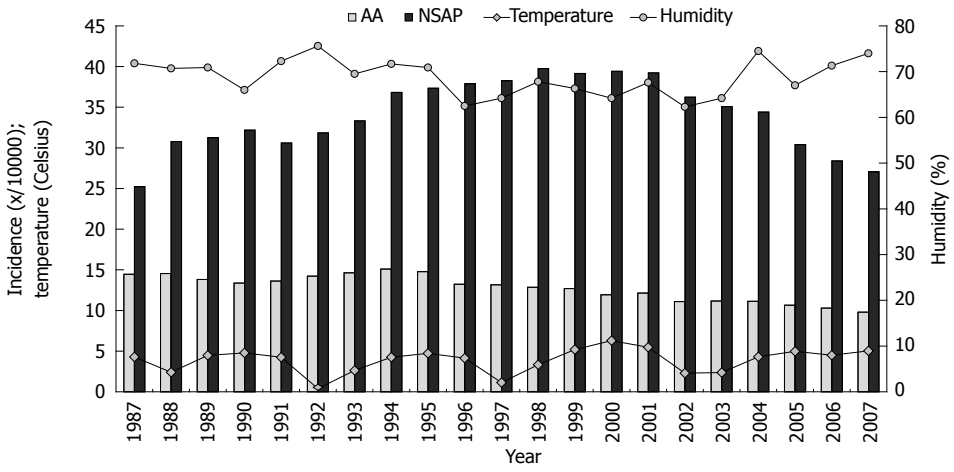

Figure 1 Temperature, humidity and incidences of acute appendicitis, and non-specific abdominal pain in 1987-2007 in Finland. AA: Acute appendicitis; NSAP: Non-specific abdominal pain.

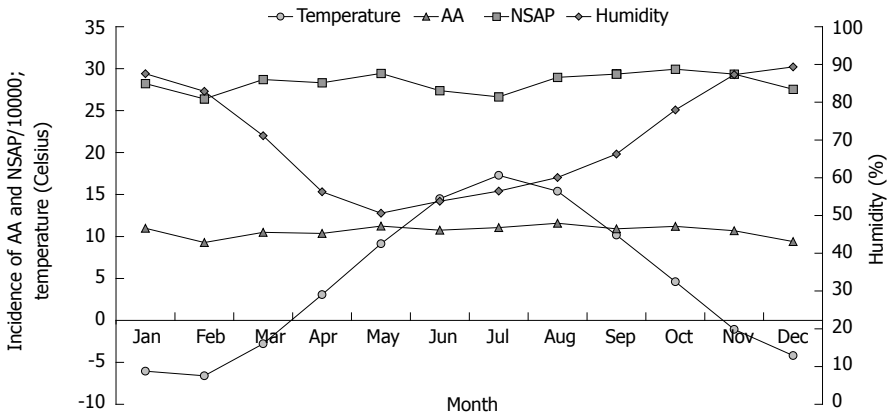

Figure 2 Acute appendicitis, non-specific abdominal pain, temperature and humidity in Finland (as mean value over the 21 years). AA: Acute appendicitis; NSAP: Non-specific abdominal pain.

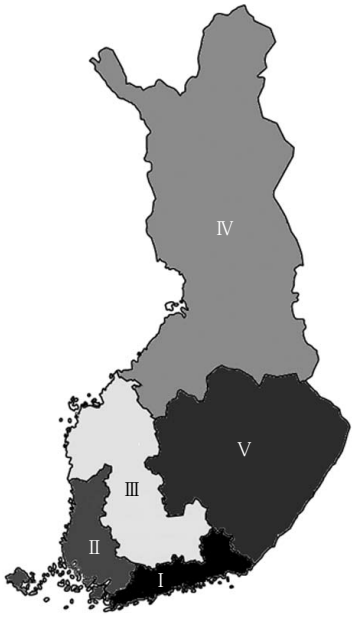

Figure 3 University hospital districts of Finland. Areas: Ⅰ: UHD of Helsinki; Ⅱ: UHD of Turku; Ⅲ: UHD of Tampere; Ⅳ: UHD of Oulu; Ⅴ: UHD of Kuopio. UHD: University hospital districts of Finland.

0.984-0.999).

DISCUSSION

In our study we found a significant association between temperature and the incidence of AA, but not for NSAP. Previously we showed that both the incidence of appendicitis and the rate of appendectomies decreased in Finland between 1987 and 2007[13]. In addition to an annual decline, there was also seasonal fluctuation in the incidence of AA. During the 21-year study period, the diagnostic accuracy for appendicitis had steadily improved. The incidence of NSAP also decreased during the last study decade.

The seasonality of several infectious and respiratory diseases is well known[15,16]. Cardiac and psychological diseases also have a seasonal behavior[17]. Furthermore, the association between AA and viral diseases has been investigated[18]. Saps *et al*[19] described the effect of seasonality on NSAP in children. The predisposing factors of appendicitis are thought to be multifactorial. The influence of seasonal variables on the incidence of AA has been discussed in many studies[11,12,20-22]. The highest incidence

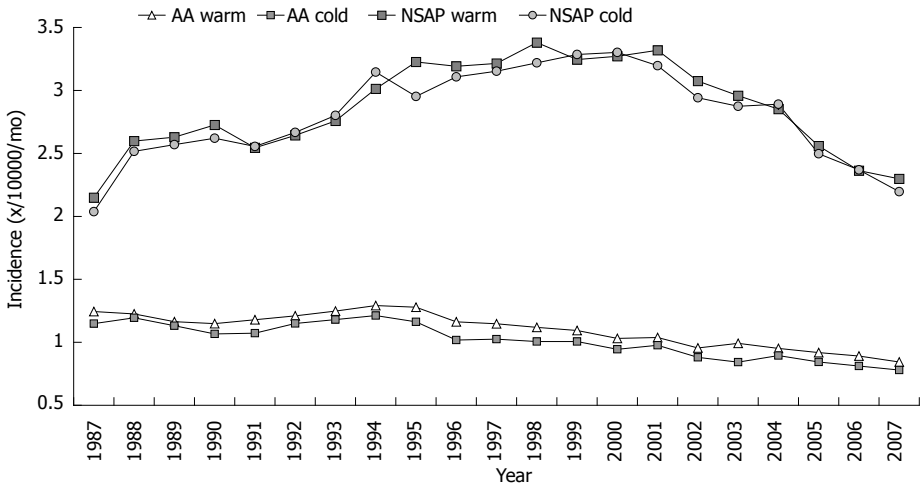

Figure 4 Incidences of acute appendicitis and non-specific abdominal pain during the warm compared to the cold periods of the year in Finland. AA: Acute appendicitis; NSAP: Non-specific abdominal pain.

of AA was found in summer and the lowest levels in winter. Moreover, the same trends in AA incidence have been shown in Nigeria[23], where the annual temperature is higher than in Finland or the United States. Unfortunately, those papers did not publish exact temperature data[11,12,20-23]. The reasons for increased incidence of AA during the warm period are not clear, although various speculations have been proposed. Examples include the effect of dehydration, lower bowel movements, infections or allergens on the reactivity of the lymphoid tissue in the appendix, and the effects of diet, humidity or changes in atmospheric pressure. Furthermore, changes in the temperature or other factors mentioned have been shown in the geographical variation of AA in the United States[20,22].

Our current study looked at a 21-year time period to investigate the fluctuation of temperature and humidity in warm and cold months, and their effects on the incidence of AA and NSAP (Figures 1, 2 and 4). Temperature had a statistically significant impaction on the incidence of AA. Although, several studies have had the same results[11,12,24,25], some additional considerations may be important. In the summertime the effect of a higher temperature on the human body is more straightforward than in winter. In Finland, winter temperatures are below zero degrees, however, people usually dress warmly. Furthermore, they spend more time inside. Therefore, the true effect of a low temperature on the human body core temperature is minor. Moreover, in a study from Nigeria, the incidence of the AA was reported to be higher during the cooler and rainy season than during the warmer season. However, the temperature in Nigeria has high levels all year round. It is possible that humidity plays a larger role in the incidence of AA in Nigeria, yet the magnitude of humidity during the rainy season in Nigeria is far greater than that in Finland[23]. Additional infections cause might further contribute to the differences between the two countries.

Another consideration is that patients with milder forms of appendicitis might not seek medical attention during the winter period[26]. On the contray, in the summer period young doctors may tend to operate on patients with lower abdominal pain with less strong indications.

Humidity had a statistically significant impact on the incidence of NSAP. The mechanism, however, continues to be unclear. There are no studies to date about seasonal variations of NSAP. It is possible that different extrinsic factors such as allergens, viral and bacterial infections might play a role during humidity changes and the etiology of NSAP. Clearly this issue needs further study.

The major strength of our study was the use of a population-based register. Moreover, the Finnish Meteorological Institute provided high-quality observational data. As a population-based retrospective study, however, there are also weaknesses. We used a discharge diagnoses in our analyses without histological confirmation. A 6% overestimation of appendicitis by surgeons has been reported[27]. In contrast, according to Kraemer *et al*[28], there was no evidence to support the assumption that the macroscopic diagnosis of appendicitis is unreliable. Furthermore, the small difference between the mean temperatures in the warm and cold period and the low mean annual temperature could be considered as a possible bias when generalizing our conclusions. However, in the reports from warmer countries (United States[22], Canada[11], Iran[12], and South Africa[16]), a similar incidence peak of AA during the warmer period was observed. Although those studies did not report exact temperatures and respective differences between the warm and cold periods, it seems reasonable to assume that higher temperatures are associated with a higher risk of developing AA. Whether temperature plays a minor or major role in the development of AA when compared to other risk factors, or whether it is an independent risk factor at all, remains to be solved in further studies.

In sum, our findings showed that AA occurs more commonly in the warmer months of the year, indicating

that the temperature may have an effect in the pathogenesis of appendicitis. No connection was seen between the temperature and NSAP.

In summary, the incidence of appendicitis but not NSAP was significantly higher during the warm time of the year. This difference remained unchanged during the whole 21-year study period. The humidity had effect on the incidence of NSAP but not on AA.

COMMENTS

Background

Acute appendicitis and nonspecific abdominal pain are two most common surgical emergencies. The etiology of both diseases still remains unclear. Many studies have reported that seasonal and weather parameters might have an effect on those diseases. Clear consensus, however, is still missing.

Innovations and breakthroughs

There are several publications about the seasonality of acute appendicitis, but no agreement exists about the impact of seasonal variables on appendicitis. To the best of our knowledge there are no studies about the seasonality of nonspecific abdominal pain.

Terminology

Appendicitis is an inflammation of the blind-ended part of the bowel connected to the cecum. Abdominal pain is a common symptom associated with transient disorders or serious disease. Diagnosing the cause of abdominal pain can be difficult because of the multifactorial nature.

Peer review

That is a good study in which the authors tried to find an answer whether temperature or humidity have an effect on acute appendicitis and nonspecific abdominal pain. Strength of the study was long examination period with large number of patients.

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RESEARCH REPORT

Acute right lower abdominal pain in women of reproductive age: Clinical clues

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Abstract

AIM: To study possible gynecological organ pathologies in the differential diagnosis of acute right lower abdominal pain in patients of reproductive age.

METHODS: Following Clinical Trials Ethical Committee approval, the retrospective data consisting of physical examination and laboratory findings in 290 patients with sudden onset right lower abdominal pain who used the emergency surgery service between April 2009 and September 2013, and underwent surgery and general anesthesia with a diagnosis of acute appendicitis were collated.

RESULTS: Total data on 290 patients were obtained. Two hundred and twenty-four (77.2%) patients had acute appendicitis, whereas 29 (10%) had perforated appendicitis and 37 (12.8%) had gynecological organ pathologies. Of the latter, 21 (7.2%) had ovarian cyst rupture, 12 (4.2%) had corpus hemorrhagicum cyst rupture and 4 (1.4%) had adnexal torsion. Defense, Rovsing's sign, increased body temperature and increased leukocyte count were found to be statistically significant in the differential diagnosis of acute appendicitis and gynecological organ pathologies.

CONCLUSION: Gynecological pathologies in women of reproductive age are misleading in the diagnosis of acute appendicitis.

Key words: Gynecological pathologies; Appendicitis; Differential diagnosis; Anamnesis; Physical examination

Core tip: Gynecological organ pathologies require to be taken into consideration when dealing with acute right lower abdominal pain in patients of reproductive age. We evaluated clinical and laboratory clues in the differential diagnosis of gynecological pathologies and acute appendicitis in patients of reproductive age. Defense, Rovsing's sign, increased body temperature and increased leukocyte count were statistically significant in the differential diagnosis of acute appendicitis and gynecological organ pathologies. In women of reproductive age with acute abdominal pain, we should also consider the probability of gynecological pathologies, therefore, gynecological anamnesis and examination should be undertaken.

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INTRODUCTION

Abdominal pain constitutes 4%-8% of adult admissions to the emergency service[1,2]. For the patient admitted with right lower quadrant abdominal pain, acute appendicitis is the most frequently considered diagnosis. Appendicitis is a common cause of acute abdominal pain in women of reproductive age (WORA) and appendectomy is the most common of all emergency operations carried out in these patients[3]. Moreover, suspected appendicitis is one of the most common surgical consultations in the outpatient or emergency room setting.

Appendicitis is an emergency situation with the highest rate of misdiagnosis, even though clear diagnosis and treatment strategies have been established for more than 100 years[4]. The inconsistency between disease severity and physical findings is greater in older patients and WORA relative to other groups. This inconsistency further increases in WORA due to gynecological pathologies mimicking acute appendicitis[5-10]. The diagnosis and management of WORA with acute appendicitis remain a difficult challenge for general surgeons and gynecologists. General surgeons may challenge gynecological pathologies and may have to intervene in these circumstances in women undergoing laparotomy with the diagnosis of acute appendicitis.

A thorough understanding of the anatomy and physiology of the abdomen is essential to properly generate a differential diagnosis and to formulate a treatment plan. Acute appendicitis can lead to unwanted complications if the diagnosis is confused or delayed. Although recent advances in surgical and diagnostic technology can be extremely helpful in certain situations, they cannot replace a surgeon's clinical judgment based on good anamnesis and physical examination.

Today, with medicine becoming more dependent on laboratory and radiological findings the merit of physical examination has decreased. It is important to understand that painstaking anamnesis and physical examination is important and may be diagnostic for many diseases, especially appendicitis. In our study, we wanted to present and emphasize how definitive anamnesis, physical examination and laboratory findings carry clues for the differential diagnosis of acute appendicitis and gynecological obstetric pathologies in WORA.

MATERIALS AND METHODS

Following Clinical Trials Ethical Committee approval, the retrospective data consisting of physical examination and laboratory findings of 290 female patients with sudden onset right lower abdominal pain who used the emergency surgery service of Adiyaman University Training and Research Hospital between April 2009 and September 2013, and underwent surgery under general anesthesia with a diagnosis of acute appendicitis were collated. The data consisted of the first findings obtained at admission and included the presence of abdominal pain, nausea, vomiting, and anorexia for anamnesis; abdominal tenderness, defense, rebound, Dunphy's sign, obturator sign, psoas sign, and Rovsing's sign for physical examination; and body temperature, leukocyte count, urine microscopy and abdominal X-ray for laboratory findings. Emergency abdominal ultrasonography (USG) and computerized tomography (CT) were not routinely performed in these patients due to an insufficiency of radiological consultation out-of-shift.

The first examination and surgery in these patients were performed by the same general surgeon. All patients underwent routine preoperative gynecological consultation. Preoperatively, the patients received a prophylactic dose of 2nd generation cephalosporin (1 g *iv*) and underwent an open approach appendectomy *via* a McBurney incision under general anesthesia. A laparoscopic approach was not performed due to technical inadequacy. Diagnosis of appendicitis and gynecological pathology was made by perioperative macroscopic evaluation. Abdominal exploration was carried out in all patients with normal appendix to exclude possible Meckel's diverticulum. Perioperative gynecological consultation was obtained for patients with gynecological pathology. Patients with previous abdominal or gynecological surgery, patients without normal menstrual cycle and pregnant patients were excluded from the study. Patients with gynecological pathologies were discharged and it was suggested that they attend a gynecology polyclinic.

Statistical analysis

All values were expressed as the mean ± standard deviation. Qualitative data were analyzed using the χ^2 test. *P* values less than 0.05 were considered statistically significant. Data were analyzed using the SPSS (Statistical Package for Social Sciences) 9.05 for Windows® statistical package.

RESULTS

The mean age of the patients was 21.4 ± 3.6 years (12-44 years). Total data for 290 patients were obtained. Two hundred and twenty-four (77.2%) had acute appendicitis, whereas 29 (10%) had perforated appendicitis and 37 (12.8%) had gynecological organ pathologies. Of the latter, 21 (7.2%) had ovarian cyst rupture, 12 (4.2%) had corpus hemorrhagicum cyst rupture and 4 (1.4%) had adnexal torsion (Table 1).

All patients had abdominal pain with right lower abdominal region tenderness and rebound as the first signs on physical examination (Figure 1). Defense, Rovsing's sign, increased body temperature (hyperpyrexia) and increased leukocyte count (leukocytosis) were found to be statistically significant in the differential diagnosis of acute appendicitis and gynecological organ pathologies (Figure 1).

All patients underwent appendectomy. Patients with normal appendix at exploration who were found to have ovarian cyst rupture underwent cauterization, ovary primary suturation and cyst excision in 16 (76.2%), 4 (19%) and 1 (4.8%) patients, respectively. Six (50%), 2 (16.7%)

Table 1 Demographic data of the patients

Parentheses	Patients (n = 290), n (%)	Age (yr)
Acute appendicitis	224 (77.2)	21 (12-44)
Perforated appendicitis	29 (10)	22 (14-42)
Ovarian cyst rupture	21 (7.2)	24 (15-38)
Corpus hemorrhagicum cyst rupture	12 (4.2)	21 (13-35)
Adnexal torsion	4 (1.4)	24 (19-30)

Data in parentheses for patients represent percentage of total number, whereas that for age indicates range.

Table 2 Treatment of patients with gynecological organ pathologies n (%)

Treatment	Ovarian cyst rupture	Corpus hemorrhagicum cyst rupture	Adnexal torsion
Cauterization	16 (76.2)	6 (50.0)	0
Primary suturation	4 (19.0)	2 (16.7)	0
Cyst excision	1 (4.8)	4 (43.3)	0
Detortion + oophoropexy	0	0	3 (75)
Oophorectomy + salpingectomy	0	0	1 (25)

and 4 (43.3%) patients with corpus hemorrhagicum cyst rupture underwent cauterization, ovary primary suturation and cyst excision, respectively. Three patients with adnexal torsion underwent detorsion and oophoropexy, whereas 1 patient underwent oophorectomy and salpingectomy (Table 2). No postoperative mortality was observed in these patients. Morbidity was observed in 11 patients (3.8%), 2 (18.2%) patients developed atelectasis and 9 (81.8%) patients developed wound infection.

DISCUSSION

Acute appendicitis is an important cause of acute abdominal pain. The incidence of appendicitis in all age groups is 7%[11,12]. The incidence of appendicitis in men and women is 8.6% and 6.7%, respectively[13]. Appendicitis is most commonly seen in subjects aged 10-30 years[14]. The mean age of the patients in our study was 21.3 ± 3.7 years. The frequency of appendicitis in males and females is equal in childhood, whereas the incidence in males increases with age with a male/female ratio of 3:2 in adulthood[15,16].

The diagnosis of acute appendicitis is made by anamnesis and clinical findings. Although it can vary with age and sex; correct diagnosis can be made in 70%-80% of patients *via* anamnesis, physical examination and laboratory findings[17-19]. Diagnostic accuracy decreases in WORA, in children and the elderly[20]. Laboratory findings and radiological examination can support the diagnosis of appendicitis, but can never rule it out. The symptoms of acute appendicitis generally follow a certain sequence and include periumbilical pain (visceral, unlocalized), anorexia, nausea and/or vomiting, right lower quadrant abdominal pain and tenderness, hyperpyrexia, and leukocytosis. These symptoms may not to be present at the same time. Physical findings suggesting appendicitis are McBurney tenderness, rebound, Rovsing's sign, Dunphy's sign, psoas sign, obturator sign and fullness and tenderness in the pelvis during digital rectal examination[17-19].

We used Dunphy's sign (increased right lower quadrant pain with coughing), obturator sign (increased pain with flexion and internal rotation of the hip), psoas sign (increased pain with passive extension of the right hip which can be elicited with the patient lying on the left side), and Rovsing's sign (increased right lower quadrant pain during palpation in the left lower quadrant) as the most common physical examination findings of appendicitis in our study[21].

The main symptoms of acute appendicitis are frequently periumbilical pain preceded by anorexia and nausea. Vomiting is generally seen later. The pain generally switches to the right lower abdominal quadrant 8 h after the initial pain[22]. The Surgical Infection Society and Infectious Diseases Society of America published guidelines that recommend the establishment of local pathways for the diagnosis and management of acute appendicitis[21,23]. According to these guidelines, the combination of clinical and laboratory findings of characteristic acute abdominal pain, localized tenderness, and laboratory evidence of inflammation will identify most patients with suspected appendicitis[21]. Our findings are shown in Figure 1.

Although the clinical presentation of periumbilical pain migrating to the right lower abdominal quadrant is classically associated with acute appendicitis, the presentation is rarely typical and the diagnosis cannot always be based on medical history and physical examination alone. Classical clinical findings of appendicitis are observed in only 60% of patients with acute appendicitis, whereas 20%-33% display atypical clinical and laboratory findings[22]. Regardless of the technological advances in the preoperative diagnosis of acute appendicitis, the correct diagnosis can only be made in 76%-92% of cases[24,25]. On the other hand, 6%-25% of operations for acute appendicitis reveal normal appendix and this number can reach 30%-40% in WORA[26-30]. Normal appendix was observed in 12.8% of patients in the present study. Diagnostic errors are common, with over-diagnosis leading to negative appendectomies and delays in diagnosis leading to perforations. Diagnostic strategies for evaluating patients with acute abdominal pain and for identifying patients with suspected appendicitis should start with a painstaking anamnesis and physical examination. All of our patients had abdominal pain with right lower abdominal region tenderness and rebound as the first signs on physical examination (Figure 1). Defense, Rovsing's sign, increased body temperature and increased leukocyte count were found to be statistically significant in the differential diagnosis of appendicitis and gynecological organ pathologies (Figure 1).

The accurate diagnosis of acute abdominal pain related to adnexal pathologies is very important for mor-

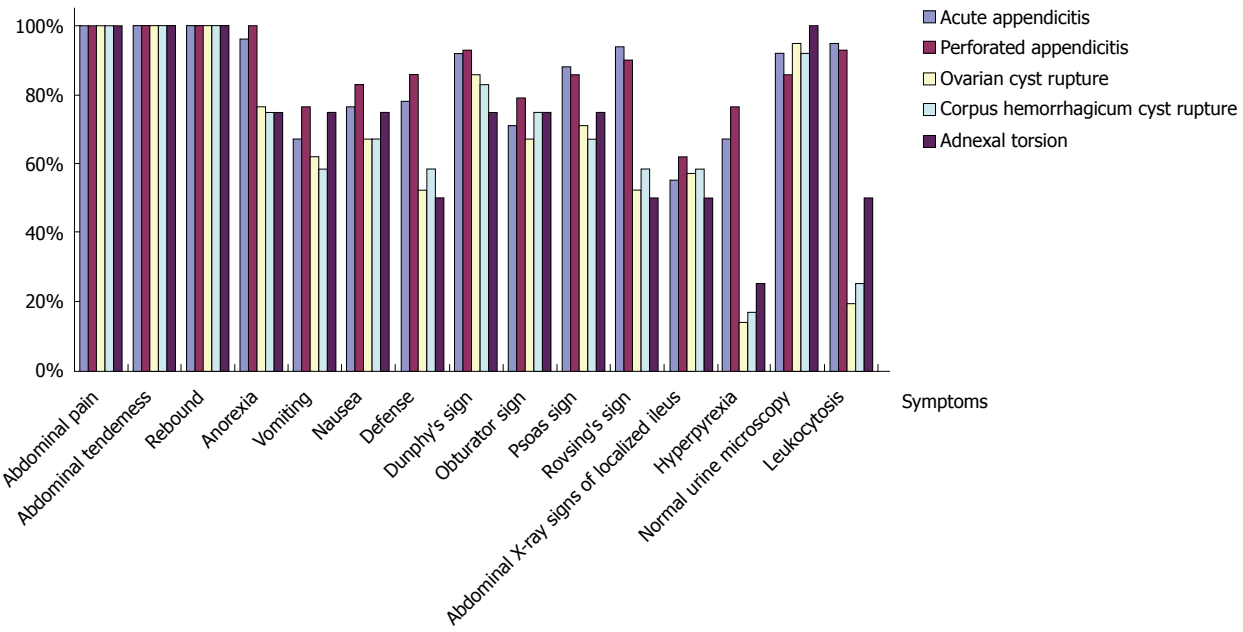

Figure 1 Clinical and laboratory data of the patients. Hyperpyrexia indicates body temperature ⩾ 37.8 ℃. Leukocytosis indicates leukocyte count > 9.000 mm^3. Defense, Rovsing's sign, hyperpyrexia and leukocytosis were different in groups with acute and perforated appendicitis; and the differences were statistically significant.

bidity and mortality. It is also crucial to choose the right treatment modality which can affect the hospitalization period and patient satisfaction. Moreover, the cost of the optimum treatment modality is important and should not be neglected. The fertility of patients can be affected when no intervention is performed for gynecological pathologies in negative appendectomy cases[31]. We observed ovarian cyst rupture, corpus hemorrhagicum cyst rupture and adnexal torsion in our study.

Pelvic pain during the ovulatory cycle may be observed due to a small amount of blood which drains from the ruptured ovarian follicle to the peritoneal cavity during ovulation. This pain is mild-to-moderate and limited, and hemoperitoneum is seldom observed with normal hemostatic parameters. Thus, there is generally no need for surgical intervention in these circumstances[32]. It is crucial to make an early correct diagnosis and to execute careful observation in patients thought to have ovarian cyst rupture if exploratory surgical intervention may result in future infertility. Adnexal masses in adolescents contain functional and physiologic cystic formations at one end of the spectrum, and serious malignant tumors at the other end. The principal clinical approach in these adnexal pathologies is to preserve organs and fertility.

Ovarian cyst rupture occurs due to benign or malignant cystic lesions of the ovaries. Cyst excision is a convenient treatment choice in young patients. It is important not to remove the whole ovary. Oophorectomy can be performed in older patients. It should be taken into consideration, that young patients with ovarian germ cell tumors may be associated with acute abdomen[5]. Hemodynamic parameters in patients with ovarian cyst rupture may be impaired due to blood loss[31,33]. Suturation, cauterization of the bleeding site or cyst excision can be performed for ovarian cyst rupture[33]. Ovarian cyst rupture was observed in 7.2% of patients in our study (Table 2). Hemodynamic parameters in these patients were stable and there was no need for blood transfusion.

Corpus hemorrhagicum cysts are one of the most common ovarian cysts. They are formed as a result of hemorrhage into the follicle cyst or corpus luteum cyst in the ovaries during the ovulation period[34-38]. The clinical signs and symptoms are variable and include patients who are asymptomatic or patients with symptoms of acute abdomen[34]. These cysts are commonly seen in a single ovary, and are rarely observed bilaterally. They are more frequently seen in patients undergoing ovulation therapy for pregnancy. They are also seen in patients with bleeding disorders and coagulation problems or those on anticoagulant treatment. They may require surgery due to intraabdominal hemorrhage as a result of rupture or torsion[36-38]. In general, bleeding can be stopped by excision of the cyst, however, sometimes the ovary needs to be removed. We observed corpus hemorrhagicum cyst rupture in 4.2% of the patients in our study (Table 1). All of these patients had stable hemodynamics and did not require blood transfusion. The patients were in their 20s and in their active reproductive period, which is in accordance with the literature[39].

Adnexal torsion is a well-known, but difficult to diagnose cause of acute abdomen due to variable clinical causes and symptoms, and involves the tuba folding up on itself. Clinical findings are similar to those of acute appendicitis[40-42]. Ovarian torsion is observed in 2%-3% of patients undergoing surgery with a diagnosis of acute appendicitis[40,41,43,44]. Ovarian torsion was observed in 1.4% of patients in the present study (Table 1). It is

observed 3-fold more frequently on the right compared with the left side[40,41]. It is relatively easy to differentiate ovarian torsion from other causes of acute abdomen *via* ultrasonography during the early period[45,46]. Adnexal torsions without symptoms are dangerous and caution should be taken in these cases. Removal of the adnex and eventual infertility risk is likely.

Excision of necrotic tissue is suggested before detorsion, due to the risk of pulmonary thromboembolism (0.2%), if vividness of the ovary is lost and a gangrene demarcation line has already formed[47,48]. In our study, we observed one patient in whom the ovary had lost its normal structure and had a necrotic appearance, and oophorectomy was performed before detorsion. Another three patients with ovarian torsion underwent detorsion and ovarian fixation (Table 2). Cohen *et al*[49] reported that torsioned, ischemic and hemorrhagic adnexa can be detorsioned laparoscopically with minimal morbidity and complete recovery of ovarian function.

The diagnosis of ectopic pregnancy is generally quick and easy following the measurement of β-hCG. We did not encounter ectopic pregnancy rupture in our study, which constitutes a significant proportion of gynecological emergencies. The reason for this may have been due to painstaking anamnesis of the patients regarding their marriage, chance of pregnancy, β-hCG values and clinical differences between ectopic pregnancy and acute appendicitis.

Abdominal ultrasonography (US) and CT are important in establishing the diagnosis of acute appendicitis preoperatively[50-52]. CT must be used to support the diagnosis and exclude other possible causes following clinical and laboratory diagnosis. Nevertheless, the ratio of negative appendectomies is higher than expected. Abdominal US, which is easy applied, inexpensive and noninvasive is the preferred method[50]. Abdominal CT is more valuable than US in this respect; the accuracy of US in the diagnosis of appendicitis is 71%-97% due to dependence on the operator and patient factors such as obesity, whereas that of CT is 93%-98%[20]. Emergency abdominal US and CT were not routinely performed in our patients due to an insufficiency of radiological consultation out-of-shift.

Leukocytosis is observed in 80%-90% of appendicitis cases, however, leukocyte number is below 18.000 mm^3 unless perforation is present[53]. Yang *et al*[54] showed a sensitivity of 85% and specificity of 31.9% for leukocyte count in appendicitis. In the present study, leukocyte counts were high in patients with acute and perforated appendicitis at 95% and 93%, respectively (Figure 1).

Currently, increased knowledge and experience, together with the development of imaging methods and laboratory techniques to evaluate patients with a gynecological emergency have facilitated the necessary general measures to minimize morbidity and mortality. When tailoring management strategies, the development and psychology of the reproductive women should be considered as well as preserving fertility which is the ultimate aim of treatment. Taking subsequent therapy into consideration, a multidisciplinary (general surgeon, gynecologist and radiologist) approach should be the basis of the management of adnexal pathologies.

In conclusion, acute appendicitis is one of the most frequent causes of acute abdomen and is also the most frequent abdominal surgical procedure. Ensuring a detailed anamnesis and medical examination is very important in the diagnosis of acute appendicitis. Laboratory findings and imaging techniques may be useful in the diagnosis. However, the diagnosis of acute appendicitis is made mainly by clinical history and clinical findings. Laboratory findings and imaging techniques support the diagnosis, but can never exclude acute appendicitis. Before establishing the diagnosis of acute appendicitis it should be remembered that gynecological pathologies may be present in WORA. Clinical findings are not always enough for definitive diagnosis and negative laparotomy is sometimes inevitable in WORA. Moreover, in view of the legal repercussions for general surgeons as a result of erroneous diagnosis and treatment, we think that adequate evaluation of the studies carried out by the emergency surgery service is important and that radiological investigations (abdominal US and CT) need to be used appropriately and sufficiently.

COMMENTS

Background

Clinical and laboratory clues in the differential diagnosis of gynecological pathologies are most likely to be confused with acute appendicitis in women of reproductive age. In these women with acute abdominal pain, the probability of gynecological pathologies should be considered, therefore gynecological anamnesis and gynecological examination should be undertaken.

Research frontiers

Evaluation of clinical and laboratory clues in the differential diagnosis of gynecological pathologies are most likely confused with acute appendicitis in women of reproductive age.

Innovations and breakthroughs

Although recent advances in medical technology can be extremely helpful in the differential diagnosis of acute abdomen, they must not replace the clinical judgment a general surgeon based upon good anamnesis and physical examination.

Peer review

In this study the authors evaluate the acute right lower quadrant abdominal pain in women of reproductive age that continues to be an open problem in general surgery. This original article is very attractive and useful.

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RESEARCH REPORT

Clinical study using novel endoscopic system for measuring size of gastrointestinal lesion

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Abstract

AIM: To verify the performance of a lesion size measurement system through a clinical study.

METHODS: Our proposed system, which consists of a conventional endoscope, an optical device, an optical probe, and a personal computer, generates a grid scale to measure the lesion size from an endoscopic image. The width of the grid scale is constantly adjusted according to the distance between the tip of the endoscope and lesion because the lesion size on an endoscopic image changes according to the distance. The shape of the grid scale was corrected to match the distortion of the endoscopic image. The distance was calculated using the amount of laser light reflected from the lesion through an optical probe inserted into the instrument channel of the endoscope. The endoscopist can thus measure the lesion size without contact by comparing the lesion with the size of the grid scale on the endoscopic image. (1) A basic test was performed to verify the relationship between the measurement error e_M and the tilt angle of the endoscope; and (2) The sizes of three colon polyps were measured using our system during endoscopy. These sizes were immediately measured by scale after their removal.

RESULTS: There was no error at α = 0°. In addition, the values of e_M (mean ± SD) were 0.24 ± 0.11 mm (α = 10°), 0.90 ± 0.58 mm (α = 20°) and 2.31 ± 1.41 mm (α = 30°). According to these results, our system has been confirmed to measure accurately when the tilt angle is less than 20°. The measurement error was approximately 1 mm in the clinical study. Therefore, it was concluded that our proposed measurement system was also effective in clinical examinations.

CONCLUSION: By combining simple optical equipment with a conventional endoscope, a quick and accurate system for measuring lesion size was established.

Key words: Lesion size; Non-contact measurement; Endoscope; Grid scale; Low level laser

Core tip: Our suggested system, which combines a conventional endoscope, several optical devices and a personal computer, can measure a lesion size by superimposing a grid scale on an image of the lesion and adjusting the width adequately according to the distance between the tip of the endoscope and the target lesion. Endoscopists can measure lesions by comparing the size of the grid scale with the size of the lesion on the monitor. After a basic performance test, the first clinical

study for 3 colon polyps was performed. In the clinical study, we confirmed that our measurement system obtained a measurement accuracy of ± 1 mm when the tilt angle of the endoscope was less than 20°.

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INTRODUCTION

In recent years, the rapid progression of endoscopic technology has enabled minimally invasive and precise screening/treatments using endoscopes in various regions of the body. Concurrently, many techniques for measuring the size of a lesion during endoscopy have been proposed. An endoscopic measurement method is strongly expected to not only improve endoscopic technology but to also assess the healing progress of lesions, to monitor organs in order to devise treatment strategies, and to analyze pathophysiology[1-6]. However, there has been no reliable measurement method used thus far. Thus, even though a lesion is discovered using an endoscope, endoscopists use only their own eyes to estimate its size[7,8]. The research and development of measurement systems has tended to focus on improving the accuracy, which increases the complexity and cost of the system. Therefore, a measurement system has yet to appear on the market.

To solve these problems, we developed a novel system for measuring lesion size. We aimed to establish and disseminate a technique based on the following five concepts: (1) simple and easy measurement; (2) inexpensive installation; (3) retention of conventional endoscopic operations; (4) accuracy on a practical level; and (5) disseminative system.

As mentioned above, we added a simplified function for measurement to the endoscope, making our system' s configuration as simple as possible. We also attempted to reduce the cost of the entire system by using an inexpensive optical device that can be directly combined with a conventional endoscope. Due to the simplicity of the measuring method and low cost of apparatus installation, our measuring system will be useful, improve current technologies, and should spread widely. The measurement of polyps/lesions is an important process because size changes constitute useful information for determining an endoscopic treatment indication. Using this simple and easy method of measurement, the information mentioned above can be readily acquired.

The aim of this paper is to introduce our newly developed lesion size measurement system, which can be combined with a conventional endoscope, to present fundamental verification regarding the distortion of an endoscopic image, and to report the first clinical study observing and measuring the polyp sizes in the colons of several patients as a comprehensive evaluation.

MATERIALS AND METHODS

Our proposed system measures the size of target lesions by displaying a grid scale (grid) on the endoscopic image. The width of the grid is automatically adjusted in real time, depending on the distance between the tip of the endoscope and the target lesion (herein referred to as the distance between objects: DBO). The shape of the grid is distorted to match the aberration of the endoscopic image. The DBO is measured without contact between the endoscope and the lesion using a laser.

This system consists of a conventional endoscope with an instrument channel, an image processor, an optical device, an optical probe, a personal computer (PC), a video mixer and a monitor (Figure 1). A laser Doppler-type blood flow meter (FLO-N1, OMEGAWAVE.INC, Tokyo, Japan) was chosen as the optical device for our system. The optical probe (diameter: φ1.8 mm) consists of two optical fibers connected to the optical device and inserted into the endoscope tip through the instrument channel. The low level laser (wavelength: 780 nm, output: 3 mW) emitted from the optical device is illuminated on a target lesion through an optical fiber inside the probe (shown as a continuous line arrow in Figure 1). The light reflected from the lesion is received by the optical device through the other optical fiber in the probe (shown as a dashed line arrow in Figure 1). The received light is converted into an electric signal inside the optical device, which is output to a PC as an analog voltage (herein referred to as REFLEX). Because the amount of received light changes according to the DBO and the optical device outputs a REFLEX equivalent to the amount of received light, there is a relationship between the REFLEX and DBO. Utilizing the characteristic that the REFLEX changes in relation to the DBO, the PC calculates the DBO on the basis of the REFLEX and then generates an adequate grid width. The shape of the grid is simultaneously distorted, corresponding to the aberration of the endoscope lens. The adjusted grid is superimposed on an original image by a video mixer and displayed on the monitor.

To assess the efficacy of our system, we examined (1) the measurement accuracy of the DBO and the displayed grid; (2) the distortion correction; (3) the size adjustment; and (4) the measurement accuracy as basic verification tests. Thereafter, a clinical test was performed in which (5) the polyp sizes in the colons of several patients were measured instead of lesion sizes.

Measurement accuracy of DBO

The endoscope was fixed at right angles with a sheet of white paper as a target object. The DBO was adjusted every 5 mm between 10 and 40 mm. The mean REFLEX was obtained at each distance for 10 s (50 ms sampling

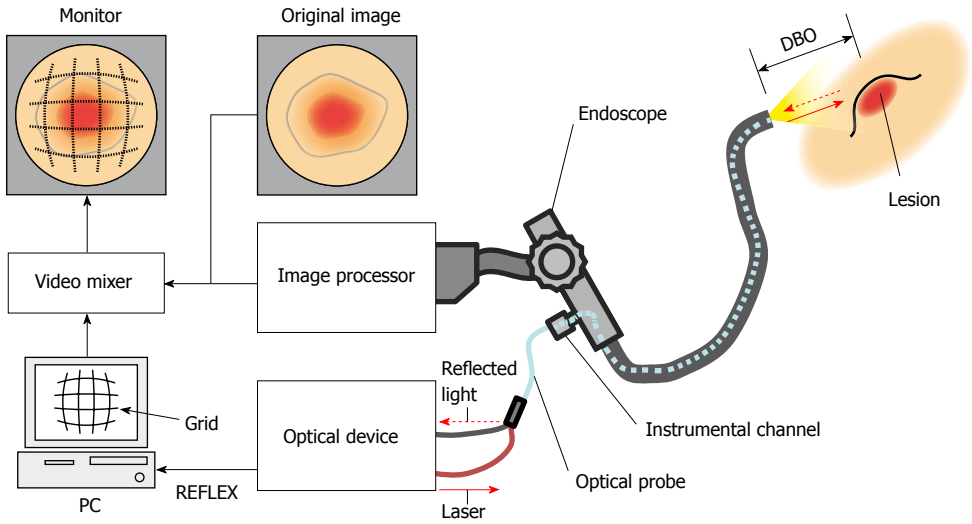

Figure 1 Diagram of the lesion size measuring system. DBO: Distance between objects.

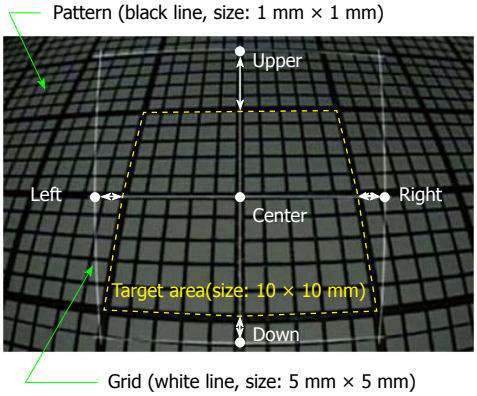

Figure 2 Endoscopic image with a 5 mm wide grid (distance between objects: 10 mm, tilt angle: 30°). The pattern of a 1 mm square (black solid lines) was observed endoscopically (DBO: 10-40 mm, tilt angle: 0°-30°), and the 5 mm wide grid (white solid lines) was superimposed on the original image. The measurement errors (white arrows) were obtained by comparing the actual area (white dashed square line, size: 10 mm square) and four spots 5 mm away from the center of the grid (Upper, Lower, Right, Left). DBO: Distance between objects.

period) in air. Then, we assumed that the relationship between the DBO value L_{DBO} and the REFLEX value V_R could be represented by Eq. (1) The values of α, calculated using power approximations, were constants that can be obtained from the data of the aforementioned experiment. $L_{DBO} = \alpha V_R^{\beta}$. Furthermore, the estimated DBO error ε_D was obtained from Eq. (2), where LT is the actual DBO. $\varepsilon_D = |L_T - L_{DBO}|/L_T \times 100$.

Distortion correction of grid shape

When an unbent grid was superimposed on the endoscopic image that was distorted by the objective lens, a measurement error occurred. Therefore, the grid shape was adjusted to match the distortion of the endoscopic image. This adjustment was accomplished by displaying 1 mm square patterns vertically on the digestive endoscope and adjusting the grid shape by superimposing the distorted patterns on the endoscopic image.

Size adjustment of the grid

Because the size of the lesion on the endoscopic image changes in relation to the DBO, it is necessary to adjust the width of the grid in real time during an endoscopic examination. Therefore, relationships between the unit pixel number corresponding to the width of the grid and the DBO were derived by displaying 1 mm square patterns vertically on the digestive endoscope, adjusting the DBO every 5 mm between 10 and 40 mm, and measuring each of the actual grid sizes on the endoscopic image.

Accuracy of grid measurement

The accuracy of the grid measurement was validated by displaying 1 mm patterns on an endoscope, superimposing the displayed pattern on a grid that was adjusted according to the DBO, and measuring the differences. The DBO was adjusted every 5 mm between 10 and 40 mm, and the tilt angle of the endoscope was adjusted every 10° between 0° and 30°. The endoscopic image in Figure 2 shows a corrected 5 mm square grid of white lines superimposed on 1 mm square patterns (DBO: 10 mm, tilt angle: 30°). The measurement error of the grid, ε_M, was obtained by comparing the target area (size: 10 mm × 10 mm, dashed yellow line) and four points (upper, lower, right, left), each of which was 5 mm away from the center of the grid. Under these conditions, the measurement errors between the grid and the pattern are shown as white arrows.

Clinical study

This study, which included a gastrointestinal clinical study, was approved by the Investigational Review Board of Fujita Health University (Aichi, Japan), and all patients gave written informed consent prior to participation. Before the clinical study, the 3 patients with colon polyps received magnesium citrate and sennoside for bowel preparation. The colon conditions of the patients did not affect the lesion size measurement because the bowel preparation resulted in a good, cleansed condition of the

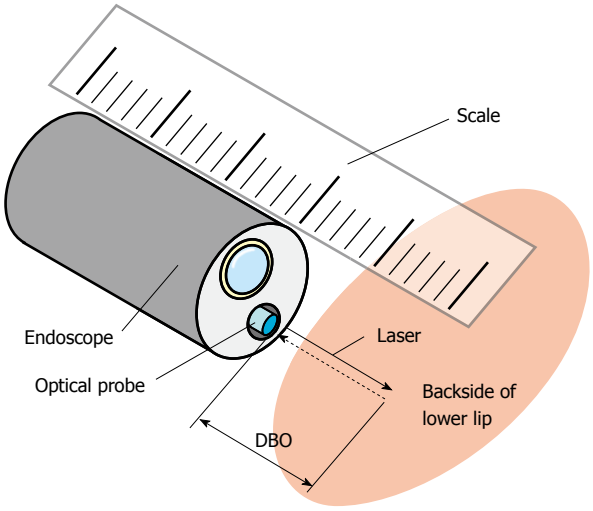

Figure 3 Image showing distance adjustment. The scale is used to measure the distance from the endoscope tip to the inside of the lower lip directly. The probe irradiates the laser on the surface of the inside of the lower lip for DBO measurement. DBO: Distance between objects.

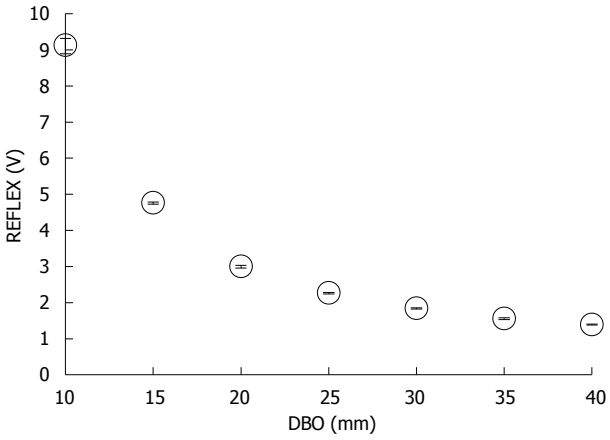

Figure 4 Difference in the amount of reflected light according to the distance between the endoscope tip and paper. The amount of reflected light decreased exponentially in proportion to the distance between the tip and paper. DBO: Distance between objects.

colon. The conventional endoscope (OLYMPUS) was used, and the endoscope and the optical probe were sterilized after each use by the same method as that used for a general endoscope.

The calibration of the measuring system was performed just before the clinical study as follows. The optical probe, which can emit a laser for DBO measurements, was inserted into the instrument channel of the endoscope. Then, the DBO was adjusted every 5 mm between 5 and 20 mm using a scale, and the REFLEX was obtained at each distance to derive Eq. (1). The shape of the grid was distorted to match the aberration of the endoscopic image. The inside of the lower lip was used as a calibration target (Figure 3).

In the clinical study, endoscopy was conducted on 3 patients to validate whether this system was able to measure the sizes of colon polyps. The endoscopists measured the polyp size, which they could observe from the front using our system. After the clinical study, 3 colon polyps were removed by hot snare and immediately measured on a scale.

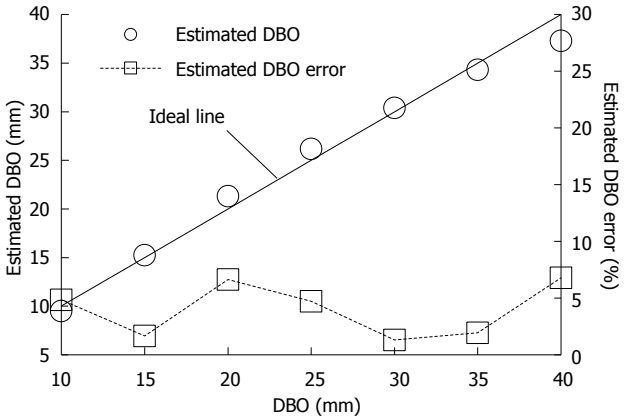

Figure 5 Comparison of the estimated distance between objects with the actual distance between objects. When the tilt angle was 0°, the estimated DBO error (mean ± SD) was 4.0% ± 2.3%. DBO: Distance between objects.

RESULTS

Measurement accuracy of the DBO

Figure 4 shows the mean REFLEX with the error range (n = 3); the figure shows that the REFLEX decreased exponentially with increasing DBO. Figure 5 shows the ideal line as a solid line using the estimated DBO (circle plots) derived from Eq. (1) (α = 47.3555, β = 0.7259) and the estimated DBO error ε_D (square plots) derived from Eq. (2). The value of ε_D (mean ± SD) was 4.0% ± 2.3%. Furthermore, the fact that the actual endoscopic examinations and treatments were performed using a sidelong view was taken into consideration. The results of our error verification of the measurements when the endoscope was tilted toward an object were as follows: 5.6% ± 1.6% at 5°, 16.6% ± 1.5% at 10°, and 48.4% ± 1.7% at 15°.

Distortion correction of grid shape

Figure 6 shows a grid shape (white lines) that was distorted to match the pattern (black lines) of the 1 mm square, as photographed by an endoscope. Images from before and after the distortion correction are shown in Figure 6A and B, respectively. The further away the grid was from the center, the more gaps occurred before the distortion correction. After performing the distortion correction, even the grids far from the center matched the distortion of the endoscopic image.

Size adjustment of the grid

Figure 7 shows the relationship between the DBO and the pixel number, which defines the size of the grid. The pixel number decreased exponentially with increasing DBO. Accordingly, when the endoscope tip approached

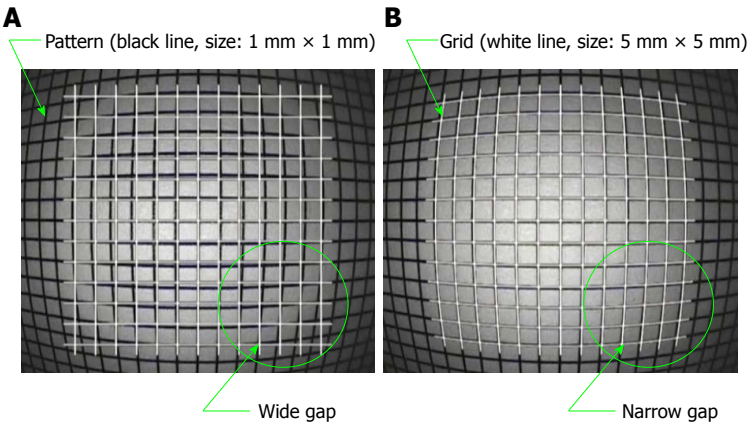

Figure 6 Distortion adjustment of grid superimposed on the original image. A: A gap occurred between the pattern (black line) and the grid (white line) before distortion processing; B: A narrow gap occurred because the grid was distorted to match the pattern.

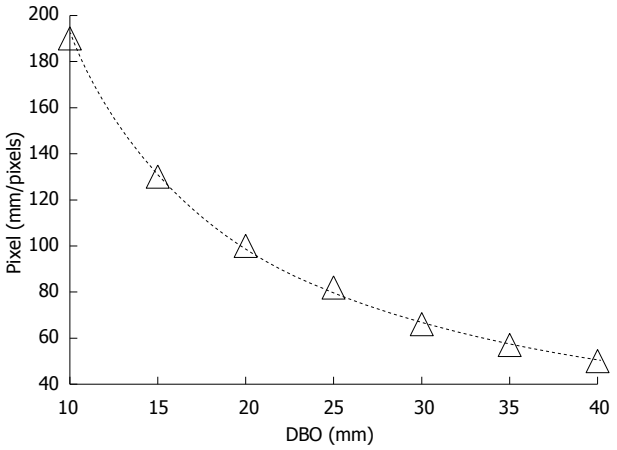

Figure 7 Relationship between the distance between objects and pixel number. As shown in this figure, when the endoscope tip approached a target, the width of the grid increased, and when the tip was retracted, the width of the grid decreased. DBO: Distance between objects.

the target lesion, the width of the grid increased, and when the tip was retracted, the width of the grid decreased. These relationships made it possible to adjust the grid width according to the DBO computed from the REFLEX in real time.

Accuracy of grid measurement

The relationships between the tilt angle of the endoscope and the ε_M values were obtained at points left and right (Figure 8A) and lower and upper (Figure 8B). There was no error at the tilt angle of 0°; therefore, the results at this angle were omitted. The value of ε_M increased with increasing tilt angle and DBO. These results also showed that the ε_M value for Upper were the largest.

For Upper, the values of ε_M (mean ± SD) were 0.24 ± 0.11 mm at 10°, 0.90 ± 0.58 mm at 20° and 2.31 ± 1.41 mm at 30°. If the tilt angle was 20° or less, the ε_M accordingly became less than 1 mm, which suggests that our system has sufficient accuracy. Therefore, in the clinical study, lesions that could be seen from a frontal view were chosen for size measurement.

Clinical study

The results of the polyp size measurements in the colon are shown in Figure 9. The left line represents the endoscopic image during the measurement using the grids (thick line every 5 mm and thin line every 1 mm) in situ; the center line is the original image, and the right line is the polyp, which is removed after the measurement using a scale *in vitro*. The measurement errors in the clinical study, ε_C, were derived from the results measured using the grid D_g and the results measured using the scale D_s and are listed in Table 1. Regarding the results, ε_C were all within 1 mm. In conclusion, even if an endoscopic image was distorted and the DBO value changed, the sizes of the lesions were accurately measured using our system.

DISCUSSION

Various methods for measuring lesion size have been proposed as endoscopic techniques have been developed. These methods can be classified into the following five categories: (1) comparative measurement; (2) image processing; (3) optical rules; (4) laser diffraction grating techniques; and (5) stereo endoscopy.

Method (1) is a comparison technique in which the size of the lesion is compared to an object of known size placed near the lesion as the measuring alternative[9-11]. It is one of the simplest measuring techniques. However, because it is necessary to place the object right next to the target lesion, there is a possibility of perforation by the object, such as biopsy forceps, of measurement instability if the object used is similar to a rubber disc, and of an unnecessary prolongation of the examination time[12].

Method (2) is an image processing technique used to correct the distortion on an endoscopic image caused by wide-angled lenses for accurate measurement[13-16]. The calibration for the measurement is easy, but this method needs further refinement to correct for the tilt angles[17].

Method (3) is based on the enlargement ratio of the image and uses the focusing mechanism of the endoscope[18]. Its operability on the endoscope is good, but the variations in focus affect the measurement's accuracy.

Method (4) is a technique that projects optical patterns toward the target using a diffraction grating or a scanning mirror, and then, the size is measured by processing the image on the basis of interferometry or triangulation[19-23]. Forthright measurement is unneces-

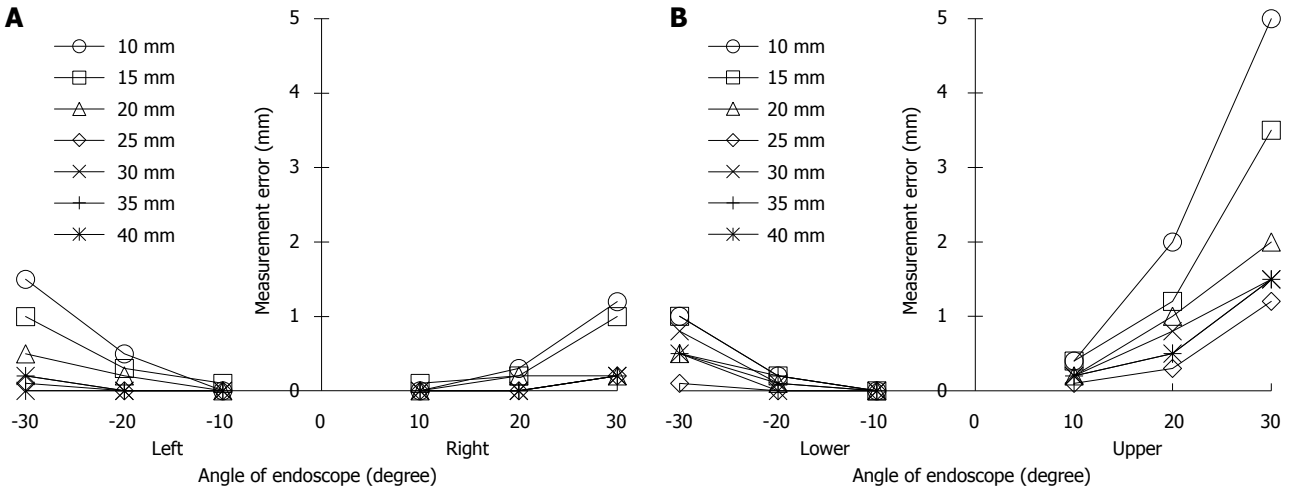

Figure 8 Evaluation results of the measurement error. A: Left and right; B: Lower and upper.

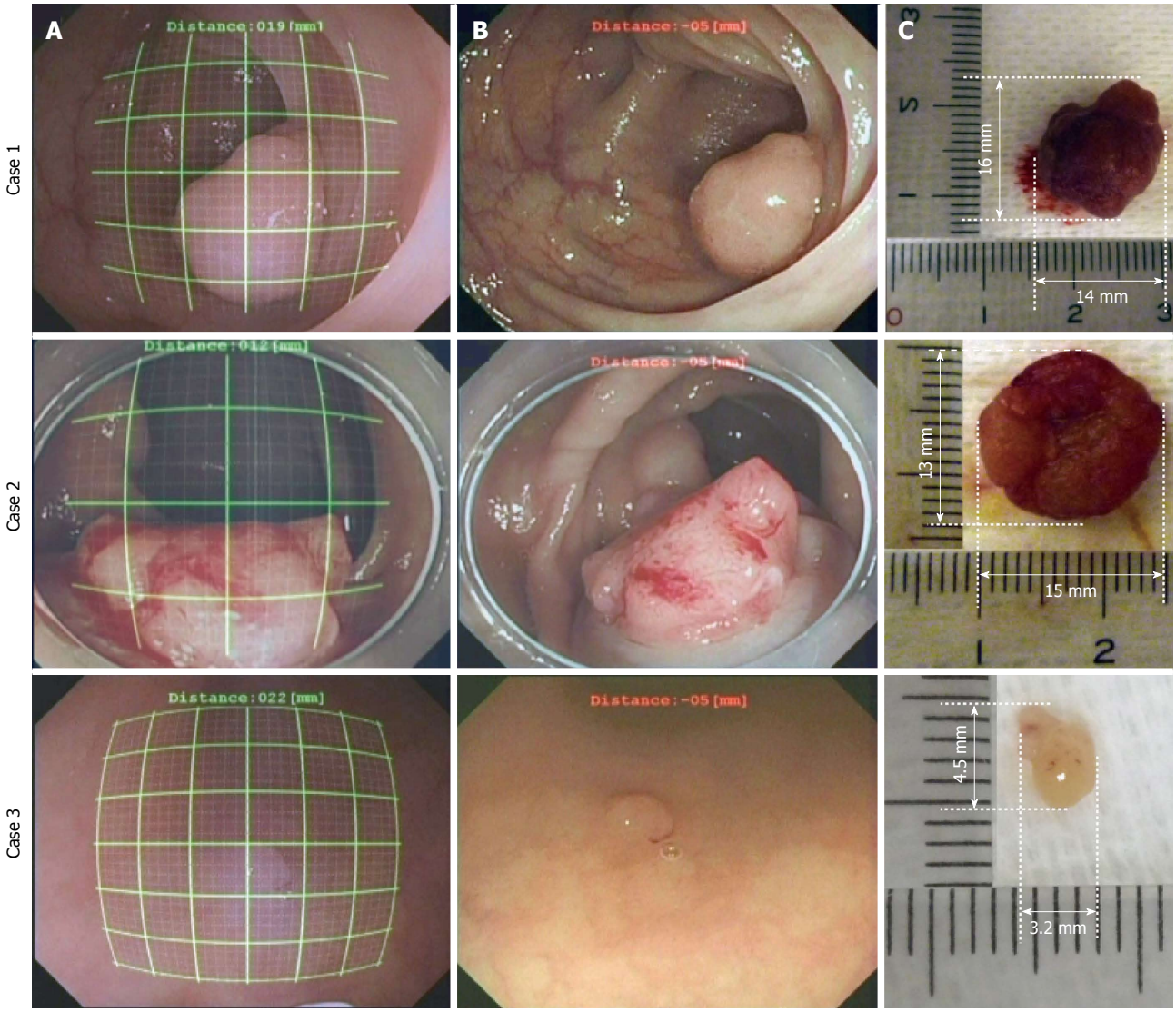

Figure 9 Results of the clinical study. A: The monitor image that endoscopists actually see to measure the polyp size; B: Original image of endoscope; C: The polyps that were removed after measurement with the polyp size measuring system.

Table 1 Measurement error for the sizes of colon polyps

Case No.	Measurement results (mm^2)		Measurement error (mm)
	Using a grid *in situ* Dg	Using a scale *in vitro* Ds	ε_C
1	13.0 × 16.0	14.0 × 16.0	1.0
2	14.0	13.0 × 15.0	1.0
3	4 × 4	3.2 × 4.5	0.8

sary, and this method has the advantage of diameter and depth measurements. However, the measurement error increases in cases when the ulcer diameter is smaller than 5 mm. There is also a concern about the initial cost of this method.

Method (5) is a technique that applies the parallax of stereo endoscope lenses and measures the three-dimensional shape of the lesion with image processing by triangulation[24,25]. This method enables the measurement of the distance and the volume of lesions; however, the initial costs and measurement times are high.

These complicated tasks slow image processing, and the high costs of implementing the methods stated above need to be improved. These problems inhibit the widespread use of these techniques in clinical practice.

However, the characteristics of our novel lesion measurement system are as follows: (1) The measurement error caused by a change in the DBO or by distortion in the endoscopic image is small; (2) Non-contact measurement is possible in real time; (3) The diameter of the probe is only 1.8 mm, and all GI endoscopes except choledochoscopes have several channels greater than 2.0 mm in diameter. Therefore, the probe can be combined with most conventional GI endoscopes[26]; (4) Special procedures for measurement are not required; and (5) Our system is feasible by simply attaching a laser for measurements to a conventional endoscope. A basic test and a clinical study were performed to verify the effectiveness of our system.

From the basic test, it was confirmed that an accurate measurement on an endoscopic image was possible in cases when the tilt angle of the endoscope tip was less than 20°. Furthermore, the measurement error decreased with decreasing tilt angle or increasing DBO.

From the clinical study, colon polyps were measured with an error of less than 1 mm. As shown in Figure 9A and B, our system worked accurately because the endoscope could be used to observe the front of a polyp that protruded from the colon wall. Furthermore, it successfully controlled the increase in measurement error due to the endoscopic tilt angle by increasing the DBO. As shown in Figure 9B, the tilt angle of the endoscope might be wider than in the case of Figure 9A. However, the polyp size was obtained at the lower part of the grid with a small error, as shown in Figure 8B. Therefore, a small error was obtained in Figure 9A.

Small polyps and a polyp spread on tissue surfaces, as shown in Figure 9C, were also measured. Because the polyps were observed from the front with the endoscope, the measurement error was small (ε_C < 0.8 mm). The measurement error increased in proportion to the increasing tilt angle, as shown in Figure 8. Therefore, when observing small lesions at close range, it is desirable to observe them from the front as directly as possible. It is difficult to measure or observe small lesions with a large tilt angle of the endoscope at close range in actual clinical settings. Consequently, lesions are most often observed under conditions that ensure a small measurement error. Thus, the sizes of lesions are inevitably measured under conditions that provide good accuracy. When the lesion is in a narrow place, such as an esophagus, where the tilt angle for grid measurement cannot be controlled, we plan on attaining grid measurement by the image processing of an endoscope image and the modification of the grid form. Although the size of the removed lesion is not the same as that of the lesion before removal, we considered that there is almost no difference between the size of a polyp that is immediately measured after removal and the size of a polyp before removal. In addition, our measurement system provides useful information for determining the lesion area and indications for endoscopic treatment, such as colon polypectomy, endoscopic mucosal resection, and endoscopic submucosal dissection.

In conclusion, our proposed system requires no special operation for the measurement of lesion size. Therefore, endoscopists can easily obtain accurate results by conventional endoscopic techniques. In addition, the necessary equipment for these measurements includes an optical device, a PC, and software, which reduces the startup costs. A measurement error of less than 1 mm is within a reasonable and permissible tolerance because the error is equivalent to or less than the errors obtained in previous studies[8,9,13,15,21,27].

In this study, the following results were obtained: (1) Our novel system for measuring lesion sizes can be combined with conventional endoscopes. Keeping the configuration of the equipment simple reduces the initial cost of the system. Although the laser Doppler blood-flow meter was used in this research, this arrangement can apply to many simple/cheap pieces of equipment in combination with an optical device; (2) Using a laser light, the measurement accuracy of the DBO was confirmed to be 4.0% ± 2.3%; (3) To improve measurement accuracy, the width of the grid had to be adjusted in real time and associated with the change in the DBO while the shape of the grid had to be distorted to match the characteristic aberrations of the endoscopic images; (4) From the verification test for the grid availability, measurement errors occurred in the range of a DBO between 10 and 40 mm and a tilt angle between 0° and 30°. The mean measurement errors for the target area (10 mm × 10 mm) were 0.24 ± 0.11 mm at a tilt angle of 10°, 0.90 ± 0.58 mm at 20° and 2.31 ± 1.41 mm at 30°. There was no error at a tilt angle of 0°. According to these results, it was confirmed that our system was able to measure accurately when the tilt angle was less than 20°. Therefore, to measure accurately, it is necessary to observe the lesions

from the front as much as possible; (5) A clinical study was performed to measure the sizes of 3 colon polyps using the grid and to then compare them with the actual sizes of the polyps after removal. The measurement error was less than 1 mm. Therefore, it was concluded that our proposed measurement system was also effective in clinical examinations; (6) This system is applicable in the following conditions: a lesion size of 16 mm (the maximum size of the results of the clinical study in case 1) or less and lesions observed from the front (tilt angle < 20°); and (7) The future aim for this technology is to improve the reliability of the measurement accuracy of our system by implementing its use in various clinical examinations.

COMMENTS

Background

Lesion size is traditionally measured by comparing the lesion to objects of known size (*e.g.*, biopsy forceps) under an endoscope. However, this method is an onerous task for a doctor as he or she must place an object near or in contact with the lesion. This new system that can measure the lesion size simply without contact has been developed, and the basic performance of our system has been verified.

Research frontiers

An endoscopic measurement method is highly expected to help in not only improving endoscopic technology but also in assessing the healing process of lesions, in monitoring organs to devise treatment strategies, and in analyzing pathophysiology. Several techniques for measuring the size of a lesion during endoscopy have been proposed, such as the comparison technique and the image processing technique. However, these techniques are not simply or easily performed by endoscopists.

Innovations and breakthroughs

A lesion size measurement system was achieved by combining a conventional endoscope with a simple/cheap optical device. Keeping the configuration of the equipment simple reduces the initial cost of the system. In addition, the system does not require any special operation.

Applications

The study's results suggest that by combining simple optical equipment with a conventional endoscope, this lesion size measurement system requires no special operation for the measurement of lesion size. Further, endoscopists can easily obtain accurate results using conventional endoscopic techniques.

Peer review

The authors have developed a new measurement system using a laser. Using this system, it is possible to measure accurately, with an error of less than 1 mm for observations from the front. Introducing this system contributes to the decision-making process during treatment and to elucidating the pathogenesis of a disease.

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RESEARCH REPORT

Single hepatocellular carcinoma ≤ 3 cm in left lateral segment: Liver resection or radiofrequency ablation?

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Abstract

AIM: To evaluate the long-term results of radiofrequency ablation (RFA) compared to left lateral sectionectomy (LLS) in patients with Child-Pugh class A disease for the treatment of single and small hepatocellular carcinoma (HCC) in the left lateral segments.

METHODS: We retrospectively reviewed the data of 133 patients with single HCC (≤ 3 cm) in their left lateral segments who underwent curative LLS (n = 66) or RFA (n = 67) between 2006 and 2010.

RESULTS: The median follow-up period was 33.5 mo in the LLS group and 29 mo in the RFA group (P = 0.060). Most patients had hepatitis B virus-related HCC. The hospital stay was longer in the LLS group than in the RFA group (8 d *vs* 2 d, P < 0.001). The 1-, 2-, and 3-year disease-free survival and overall survival rates were 80.0%, 68.2%, and 60.0%, and 95.4%, 92.3%, and 92.3%, respectively, for the LLS group; and 80.8%, 59.9%, and 39.6%, and 98.2%, 92.0%, and 74.4%, respectively, for the RFA group. The disease-free survival curve and overall survival curve were higher in the LLS group than in the RFA group (P = 0.012 and P = 0.013, respectively). Increased PIVKA-Ⅱ levels and small tumor size were associated with HCC recurrence in multivariate analysis.

CONCLUSION: Liver resection is suitable for single HCC ≤ 3 cm in the left lateral segments.

Key words: Small hepatocellular carcinoma; Left lateral segment; Radiofrequency ablation; Liver resection; Tumor recurrence; Survival

Core tip: Many papers have reported the relative outcomes between liver resection and radiofrequency ablation, but here we selected patients with small and single hepatocellular carcinoma (HCC) in the left lateral segments. The present study showed that the disease-free survival curve and the overall survival curve were higher in the left lateral sectionectomy (LLS) group than in the radiofrequency ablation (RFA) group for

those patients. However, the hospital stay was longer for the LLS group than for the RFA group. We conclude that liver resection is suitable for single HCC ≤ 3 cm in the left lateral segments.

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INTRODUCTION

Screening programs for patients with hepatitis B virus (HBV) have led to increasingly earlier diagnoses of hepatocellular carcinoma (HCC)[1]. Recent progress in imaging modalities has also facilitated increased diagnosis of small HCC in endemic areas, such as South Korea.

Following the Milan criteria (single HCC ≤ 5 cm or up to 3 nodules < 3 cm), the best treatment for HCC is liver transplantation, but this procedure is limited by the scarcity of donors[2]. Surgical resection is thus considered the first-choice treatment for patients with early stage HCC, and offers a 5-year-survival rate of over 50%[3]. Percutaneous ablation is usually reserved for patients who are not candidates for surgery owing to impaired liver function or co-morbidity, or for those who refuse surgery[4].

The American Association for the Study of Liver Diseases (AASLD) recommends percutaneous radiofrequency ablation (RFA) for three or fewer 3 cm or smaller early-stage HCCs, or 2 cm or smaller very-early-stage HCCs with complications such as portal hypertension[5]. Currently, RFA competes with liver resection and liver transplantation as the primary treatment for small HCC. RFA has attracted the greatest interest due to its advantages over liver resection, including less destruction of normal liver tissue, lower cost, no necessity for blood transfusion, lower complication rate, and shorter hospital stay[6,7]. However, there is still debate with regard to whether percutaneous RFA or liver resection is the most suitable therapy for small HCC or certain tumor locations. Several randomized controlled trials and many non-randomized controlled trials have been published in an attempt to address this question.

The purpose of this study was to retrospectively evaluate the long-term results of percutaneous RFA compared with left lateral sectionectomy (LLS) in patients with Child-Pugh class A liver cirrhosis for the treatment of single and small HCC in the left lateral segment.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the data of 133 patients with HCC in their left lateral segments (S2 and/or S3) who underwent curative LLS or percutaneous RFA at Samsung Medical Center between January 2006 and June 2010. All patients had a single tumor of 3 cm or less in diameter without extrahepatic metastasis detected during pre-treatment imaging such as 3-phase computed tomography (CT) and/or dynamic magnetic resonance imaging (MRI). Enrolled patients had Child-Pugh class A liver cirrhosis or non-cirrhotic livers and no previous history of surgical resection or locoregional therapy for HCC. The diagnosis of HCC was based on pathologic confirmation, elevated serum α-fetoprotein (AFP) (≥ 400 ng/mL) with radiologic findings, or at least two coincidental radiologic findings compatible with HCC in high-risk patients[8]. Patients younger than 18 years or with tumor size more than 3 cm, tumor in segments other than the left lateral segments (S2 or S3), other pathological or radiological malignancy in liver, or those lost to follow-up after hepatectomy or RFA were excluded from this study. The demographic and preoperative laboratory data of all patients were retrieved from electronic medical records (EMR) and were retrospectively reviewed. None of the patients in either group received postoperative adjuvant therapy before recurrence was detected.

Radiofrequency ablation

Patients with small HCC in their left lateral segments were screened by planning ultrasonography to determine the feasibility of percutaneous RFA[9]. If the tumor was located at risk locations for RFA, such as superficially and adjacent to the hepatic vein, portal vein, and/or heart, liver resection was preferentially recommended. All RFA procedures were performed percutaneously under real-time ultrasound guidance with conscious sedation. Procedures were performed on an inpatient basis by one of six radiologists, each of whom had at least 7 years of experience performing this procedure by the end of the study period. We used either internally cooled, multi-tined expandable, or perfusion electrode systems according to temporal availability or operator preference. When we used internally cooled electrodes, we started at 50 W and continuously increased the power during the initial 2 min to minimize the popping phenomenon. All patients were treated with 2% lidocaine hydrochloride at the puncture site and intravenous drip infusion of 50 mg pethidine hydrochloride mixed with 50 mL of 5% dextrose water. Patient cardiovascular and respiratory systems were continuously monitored during the procedure. Our therapeutic strategy for RF ablation was to obtain at least 0.5 cm of the normal liver surrounding the tumor as a tumor-free margin insofar possible[10].

Surgery

Before surgery, each patient underwent conventional liver function tests and indocyanine green retention rate measurements at 15 min (ICG-R15). Preoperative tests of liver function included serum bilirubin, transaminases, alkaline phosphatase, albumin, and prothrombin time. The levels of AFP and protein induced by vitamin

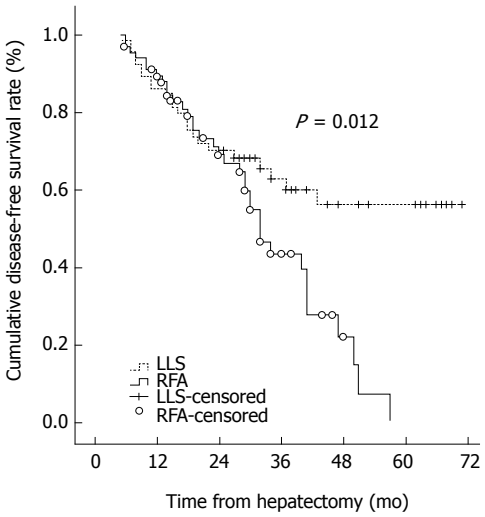

Figure 1 Disease-free survival. The 1-, 2-, and 3-year disease-free survival rates were 80.0%, 68.2%, and 60.0%, respectively, in the LLS; and 80.8%, 59.9%, and 39.6%, respectively, in the RFA group. The disease-free survival curve was better for the LLS group than for the RFA group (P = 0.012). LLS: Left lateral sectionectomy; RFA: Radiofrequency ablation.

K absence/antagonism-Ⅱ (PIVKA-Ⅱ) were also measured in all patients. Selection criteria for the liver resection procedure in the left lateral segments depended on bulging tumor in a superficial site and/or location close to vessel and heart. Child-Pugh class C, severe comorbidity, and distant metastasis were considered contraindications for hepatectomy. Standard operative techniques for hepatectomy were used[11].

Surveillance after treatments

Patients were followed every 2-3 mo postoperatively. Follow-up included physical examination, serum AFP, PIVKA-Ⅱ, liver function tests, and chest X-ray. Helical dynamic triple phase CT was performed every 3 mo for the detection of local tumor progression, new intrahepatic recurrence, and extrahepatic metastasis or when recurrence was suspected. MRI and/or positron emission tomography (PET) scan were performed when CT was not definitive. Diagnoses of HCC recurrence were based on CT and/or MRI. Needle biopsies of recurrent tumors were not performed.

Statistical analysis

Continuous variables were presented as median and range and were compared by the Mann-Whitney U test. Categorical variables were compared by Fisher's exact test, as appropriate. Disease-free survival rates and overall survival rates were calculated by the Kaplan-Meier method. Differences between the curves were assessed using the log-rank test. Variables that showed statistical significance in univariate analyses were included in multivariate analyses using Cox proportional hazard models. A value of $P < 0.05$ was considered statistically significant. All data were analyzed using SPSS statistical software (Ver 21.0; SPSS Inc., Chicago, IL, United States).

RESULTS

Patients

A total of 133 patients with HCC ≤ 3 cm in their lateral segments (S2 and S3) were reviewed. Sixty-six patients were initially treated with surgical resection, such as LLS, while 67 patients were initially treated with percutaneous RFA. The baseline characteristics of all patients are outlined in Table 1. The median follow-up period was 33.5 mo (range, 1-66 mo) for LLS and 29 mo (range, 1-73 mo) for percutaneous RFA (P = 0.060). Most patients had HBV-related HCC, and the proportion of HCV-related HCC was higher in the percutaneous RFA group than in the LLS group (25.4% *vs* 6.2%). The age, serum AST levels, and ICG-R15 were higher in the RFA group than in the LLS group, but white blood cell counts, serum hemoglobin levels, platelet counts, serum albumin levels, PIVKA-Ⅱ levels, and tumor size were higher in the LLS group. General liver function was better in the LLS than in the RFA group despite the Child-Pugh class A status of patients. The median hospitalization of the LLS group was 8 d (range, 3-68 d), as opposed to 2 d (range, 2-26 d) for the RFA group. The hospital stay was longer in the LLS group than in the RFA group (P < 0.001).

Outcomes

At last assessment, 23 patients in the LLS group and 35 in the RFA group had developed tumor recurrence. The 1-, 2-, and 3-year disease-free survival and overall survival rates were 80.0%, 68.2%, and 60.0%, and 95.4%, 92.3%, and 92.3%, respectively, for the LLS group; and 80.8%, 59.9%, and 39.6%, and 98.2%, 92.0%, and 74.4%, respectively, for the RFA group. The disease-free survival curve and overall survival curve were higher in the LLS group than the RFA group (P = 0.012 and P = 0.013, respectively) (Figures 1 and 2). Eleven patients in the RFA group developed local tumor progression. The 1-, 2-, and 3-year local tumor progression rates in the RFA group were 90.9%, 85.1%, and 82.3%, respectively. For HCC less than or equal to 2 cm, the 1-, 2-, and 3-year disease-free survival and overall survival rates were 75.8%, 69.7%, and 50.3%, and 97.0%, 88.2%, and 88.2%, respectively, in the LLS group; and 78.6%, 60.5%, and 35.3%, and 97.4%, 94.3%, and 80.9%, respectively, in the RFA group (P = 0.183 and P = 0.074, respectively). There were no statistically significant differences in disease-free survival and overall survival between the RFA group and the LLS group in patients with HCC ≤ 2 cm.

Tumor recurrence and treatment

In the RFA group, 35 patients had intrahepatic recurrence and 10 patients showed concurrent intrahepatic and systemic recurrence. None developed only extrahepatic recurrence. Of these 35 patients, 12 were treated with a second percutaneous RFA and 15 with transarterial chemoembolization (TACE). Six patients were treated

Table 1 Baseline characteristics of patients

Characteristics	LLS (*n* = 66)	RFA (*n* = 67)	*P* value
Gender-male	48 (72.7)	52 (77.6)	0.514
Age (yr)	55 (27-76)	59 (39-85)	0.002
BMI	23.5 (17.8-33.4)	23.6 (18.5-32.0)	0.374
Etiology			0.014
HBV	51 (78.5)	44 (65.7)	
HCV	4 (6.2)	17 (25.4)	
Alcoholic	2 (3.1)	2 (3.0)	
NBNC	4 (6.2)	4 (6.0)	
Others	4 (6.2)	0 (0)	
WBC (/μL)	5345 (2600-8950)	4000 (2000-11000)	0.007
Hemoglobin (g/dL)	14.2 (10.8-17.7)	14.0 (8.0-17.0)	0.001
Platelet (/μL)	149500 (51000-276000)	103000 (50000-257000)	0.000
INR	1.1 (0.9-1.3)	1.00 (1.0-2.0)	0.000
Albumin (g/dL)	4.3 (3.5-4.9)	4.0 (3.0-5.0)	0.000
Total bilirubin (mg/dL)	0.7 (0.2-1.7)	1.0 (0.2-2.0)	0.538
AST (IU/L)	33 (16-95)	38 (12-124)	0.007
ALT (IU/L)	30 (10-162)	35 (8-138)	0.477
ALP (IU/L)	78 (35-176)	83 (45-189)	0.392
Creatinine (mg/dL)	0.91 (0.50-1.27)	0.88 (0.46-2.64)	0.507
ICG-R15	10.5% (2.3%-24.9%)	16.8% (3.3%-45.2%)	0.000
AFP (ng/mL)	28.5 (1-7102)	20.0 (2-5652)	0.323
PIVKA-Ⅱ (mAU/mL)	25 (3-500)	18 (9-500)	0.011
Tumor size (cm)	2.1 (0.8-3.0)	1.8 (1.0-2.9)	0.035

Data are presented as "*n* (%)" or "median (range)". LLS: Left lateral sectionectomy; RFA: Radiofrequency ablation; BMI: Body mass index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Non-B, non-C; WBC: White blood cells; INR: International normalized ratio; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; ICG-R15: Indocyanine green retention rate at 15 min; APF: Alpha-fetoprotein; PIVKA-Ⅱ: Protein induced by vitamin K absence/antagonism-Ⅱ.

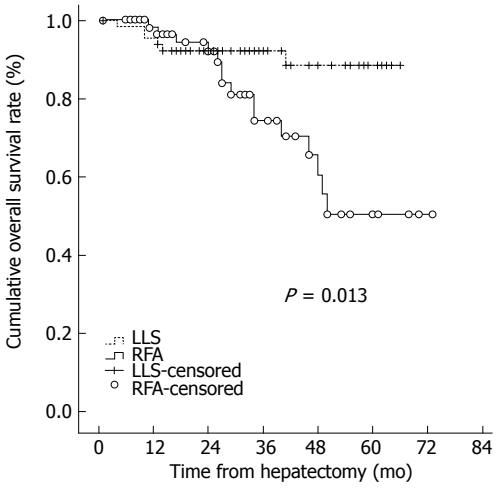

Figure 2 Overall survival. The 1-, 2-, and 3-year overall survival rates were 95.4%, 92.3%, and 92.3%, respectively, in the LLS group; and 98.2%, 92.0%, and 74.4%, respectively, in the RFA group. The survival curve for the LLS group was higher than for the RFA group (*P* = 0.013). LLS: Left lateral sectionectomy; RFA: Radiofrequency ablation.

with simultaneous RFA and TACE. Two patients were treated with surgical resection because HCC recurred in segment 6 in one patient and segment 8 in the other patient with good liver function. Of the 23 patients with recurrence in the LLS group, 17 patients had intrahepatic recurrence and two had systemic recurrence, while four patients had concurrent intrahepatic and systemic recurrence. Of the recurrent patients in the LLS group, eight were treated with TACE, four with RFA, and five with simultaneous TACE and RFA, while four patients received no treatment, and two patients were treated with a second liver resection. The 3-year overall survival rate was 93.3% in the LLS group and 74.4% in the RFA group ($P = 0.018$). The overall survival curve was higher for the LLS group than for the RFA group (Figure 2, $P = 0.013$).

Risk factors for tumor recurrence

Among all the variables, treatment allocation (such as RFA), platelet counts, serum albumin, ICG-R15, PIVKA-Ⅱ levels, and tumor size were found to be significant risk factors of disease-free survival by univariate analysis (Table 2). Multivariate Cox regression hazard regression analyses showed that PIVKA-Ⅱ levels (OR = 1.005; 95%CI: 1.001-1.009, $P = 0.010$) and tumor size (OR = 0.915; 95%CI: 0.853-0.981, $P = 0.012$) were significant prognostic factors for disease-free survival.

DISCUSSION

Many studies have reported that surgical resection reduces the risk of recurrence of HCC, but failed to demonstrate any difference in the overall survival following resection versus RFA in patients with small HCC[4,12-14]. Our study showed that liver resection was associated with a significantly lower risk of both death and recurrence than was RFA in patients with small HCC in the left lateral segments. This difference is particularly evident in the long term. The curves of disease-free survival and overall survival in the LLS group were higher

Table 2 Risk factors for hepatocellular carcinoma recurrence by univariate analysis

Risk factors	OR	95%CI	*P* value
Group-RFA	1.934	1.141-3.277	0.014
Gender-female	0.536	0.264-1.090	0.085
Age	1.015	0.991-1.040	0.217
BMI	1.066	0.972-1.169	0.177
WBC	0.924	0.781-1.093	0.354
Hemoglobin	0.900	0.773-1.047	0.171
Platelet	0.993	0.988-0.998	0.008
INR	0.730	0.019-27.673	0.865
Albumin	0.407	0.255-0.650	0.000
Total bilirubin	0.970	0.539-1.746	0.920
AST	1.008	0.996-1.019	0.196
ALT	1.002	0.992-1.011	0.736
ALP	1.001	0.993-1.009	0.816
Creatinine	1.511	0.490-4.655	0.473
ICG-R15	1.048	1.015-1.082	0.004
AFP	1.000	1.000-1.000	0.980
PIVKA-Ⅱ	1.004	1.001-1.007	0.017
Tumor size	0.952	0.907-1.000	0.050

OR: Odds ratio; CI: Confidence interval; RFA: Radiofrequency ablation; BMI: Body mass index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NBNC: Non-B, non-C; WBC: White blood cells; INR: International normalized ratio; AST: Aspartate transaminase; ALT: Alanine transaminase; ALP: Alkaline phosphatase; ICG-R15: Indocyanine green retention rate at 15 min; APF: Alpha-fetoprotein; PIVKA-Ⅱ: Protein induced by vitamin K absence/antagonism-Ⅱ.

than in the RFA group, despite the RFA group showing low liver function *via* such metrics as high ICG-R15, low platelet count, and low serum albumin levels. However, all RFA patients were treatable with liver resection. This study reconfirmed that liver resection is associated with a reduced recurrence rate in HCC located in the left lateral segments and revealed that resection yielded longer overall survival than did RFA.

Treating hepatocellular carcinoma in patients with chronic liver disease has always presented a challenge because of the clinical complexity of managing these patients and the potential risks associated with postoperative complications. The risk factors for tumor recurrence after treatment include tumor size, insufficient safety margin, multi-nodular tumor, and tumor location[15]. Liver resection in patients with resectable HCC who have normal liver function and are in good general condition is still considered the gold standard therapy for delivering curability[12,16]. However, patients with central HCC are not usually good candidates for surgical resection because of the risk of additional injury to normal liver tissue and blood loss, which may induce further complications and negatively impact treatment outcome. RFA, however, preserves the liver parenchyma, and has a low risk of blood loss. In recent years, it has been possible to reduce perioperative mortality to less than 0.5% depending on the extent of resection and hepatic reserve[11]. The improved outcome is primarily due to advances in surgical and radiologic techniques, perioperative care and more cautious patient selection[17]. Surgical resection of tumors located in the left lateral segments is considered a safe procedure because it is easily practicable from a technical standpoint, as well as due to ease of accessibility. Recently, laparoscopic LLS has been established as a safe and feasible standard treatment option for malignant liver tumors at some specialized centers[18]. In the present study, some patients were treated with laparoscopic LLS. However, the follow-up period of those patients was too short, and we did not compare the laparoscopic LLS group with the RFA group. We will continue to collect data on laparoscopic LLS.

The RFA procedure can be performed under conscious sedation and most patients only require a short hospitalization after the procedure. There is general consensus that complete response to percutaneous RFA therapy in patients with tumors of less than or equal to 3 cm is associated with improved outcome[13,14,19]. Whether RFA or surgical resection is the better treatment option for small HCC has been debated since RFA was recommended as a treatment option in the 2005 practice guidelines issued by the AASLD[20]. Two recent meta-analyses reached significantly different conclusions, mainly because the majority of the data were obtained from non-randomized controlled trials and the overall level of clinical evidence was low[14,19]. The conclusions reported from two randomized-controlled trials were also contradictory[16,21]. Another recent randomized controlled study showed that percutaneous RFA may provide therapeutic effects similar to those of liver resection in patients with small HCC[13]. However, outcomes of RFA and resection have not been compared for left lateral segments. In this study, we therefore limited our objectives to patients with HCC $\leqslant$ 3 cm in left lateral segments.

Compared to surgical resection, percutaneous RFA is more likely to be incomplete for the treatment of small HCCs located at specific sites of the liver, such as those with bulging tumor, as well as the adjacent regions of the heart and diaphragm, and major vessels. Open or laparoscopic surgery may be the better choice in these patients. HCC mainly disseminates through the portal and hepatic veins. The tumor dissemination can invade the tributaries of the portal branches and shed tumor emboli in the neighboring branches of the same liver segment[22]. Liver resection has the advantage of complete excision of tumor tissue and hepatic parenchyma around the tumor, which might contain undetectable intrahepatic metastases and microvascular invasion[23]. Therefore, liver resection with safe tumor-free margins has better results than RFA with respect to tumor recurrence.

In this study, local recurrence was found to be more frequent after RFA than LLS, as eleven patients in the RFA group developed local tumor progression, whereas none developed it in the LLS group. This may be a result of the safety margin of RFA being narrower than that of LLS. LLS removes the entire left lateral segment containing the primary tumor and venous tumor thrombus[24,25], and the clearance of tumors and any potential sites of microscopic disease will be more complete in these patients. Local recurrences after RFA may be attributed to insufficient ablation of the primary tumor

and/or the presence of tumor venous invasion in the adjacent regions of the liver. However, our study showed that the LLS group may have poor prognostic factors, such as microvascular invasion because patients with vessel-adjacent tumors were treated by surgical resection.

This study suggests that disease-free and overall survival rates following liver resection were superior to those following RFA. We therefore consider RFA to be significantly worse than LLS in the long-term. Percutaneous RFA was demonstrated to have an advantage over liver resection in terms of shorter hospitalization length. We suspected that some factors were correlated with early tumor recurrence after treatment, independent of the treatment strategy, and such factors of early recurrence were identified in this study.

In our study, patients who chose RFA as the first treatment modality were significantly older than those who underwent liver resection. Older patients may choose RFA because they more commonly have comorbidities that make liver resection unfeasible. In addition, RFA is less invasive and has lower rates of complications and lower costs, and higher repeatability when recurrence occurs[7]. The choice of RFA by older patients is consistent with data from a large, nationwide cohort study from Japan[26].

Our study had several limitations. First, it was a retrospective study. Thus, the present study was inherently flawed by a selection bias evident in the differences in tumor, etiology, and liver functions. Second, we did not assess the histopathologic diagnosis of HCC in the RFA group. Patients with poorly differentiated HCC have a poorer outcome than patients with well to moderately differentiated HCC after percutaneous RFA[27], and our study showed that a small tumor size was associated with risk factors for tumor recurrence. It is possible that HCC in the RFA group was associated with benign liver diseases, such as nodular liver cirrhosis or inflammatory pseudotumors, which may have influenced the overall survival and recurrence rates found in this study. Third, data on liver function during the follow-up was absent, which precluded assessment of the relationship between liver function and the choice of treatment at recurrence. For HCC, the influence of the first treatment is considered to be smaller than for other primary malignant diseases, because liver function significantly affects recurrence rate. Fourth, the absence of recurrence was not verified by pathologic examination, which suggests that the reported local recurrence rates for RFA may have been underestimated.

We created groups with three uniform criteria: tumor size $\leqslant$ 3 cm, Child-Pugh class A, and tumor located in left lateral segments, with the aim of producing a focused study and contributing to the current discussion on the management of HCC. We believe that despite the inherent drawbacks of our study design, our results are useful given the current lack of reliable data derived from well-designed randomized controlled trials.

In conclusion, liver resection is suitable in single HCC $\leqslant$ 3 cm in the left lateral segments. A future prospective multi-center study of the local recurrence rates of small HCC stratified according to tumor location is needed to provide clinically useful data on this issue.

COMMENTS

Background

Liver resection is considered the first-choice treatment for patients with early stage hepatocellular carcinoma (HCC), but recently radiofrequency ablation in patients with small HCC achieved the similar outcomes of surgical liver resection. Many studies have reported the efficacy between liver resection and radiofrequency ablation.

Research frontiers

Nobody recommend the liver resection or radiofrequency ablation in small HCC patients. In addition, all studies do not consider the location of tumor and the extent of surgical resection.

Innovations and breakthroughs

This study has a high value because this was the first study that evaluated patients with HCC located in left lateral segments. Present study showed that the disease-free survival curve and overall survival curve were higher in the left lateral sectionectomy group than in the radiofrequency ablation group.

Applications

Present study suggests that liver resection is suitable for single HCC $\leqslant$ 3 cm in the left lateral segments.

Peer review

The authors compared the outcome of liver resection (left lateral sectionectomy, left lateral sectionectomy) *vs* radiofrequency ablation for single HCC $\leqslant$ 3 cm in left lateral segments. The paper is relevant to this journal, and in general well written.

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RESEARCH REPORT

YouTube as a source of patient information on gallstone disease

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Abstract

AIM: To investigate the quality of YouTube videos on gallstone disease and to assess viewer response according to quality.

METHODS: A YouTube search was performed on September 18, 2013, using the keywords "gallbladder disease", "gallstone disease", and "gallstone treatment". Three researchers assessed the source, length, number of views, number of likes, and days since upload. The upload source was categorised as physician or hospital (PH), medical website or TV channel, commercial website (CW), or civilian. A usefulness score was devised to assess video quality and to categorise the videos into "very useful", "useful", "slightly useful", or "not useful". Videos with misleading content were categorised as "misleading".

RESULTS: One hundred and thirty-one videos were analysed. Seventy-four videos (56.5%) were misleading, 36 (27.5%) were slightly useful, 15 (11.5%) were useful, three (2.3%) were very useful, and three (2.3%) were not useful. The number of mean likes (1.3 ± 1.5 *vs* 17.2 ± 38.0, $P = 0.007$) and number of views (756.3 ± 701.0 *vs* 8910.7 ± 17094.7, $P = 0.001$) were both significantly lower in the very useful group compared with the misleading group. All three very useful videos were PH videos. Among the 74 misleading videos, 64 (86.5%) were uploaded by a CW. There was no correlation between usefulness and the number of views, the number of likes, or the length. The "gallstone flush" was the method advocated most frequently by misleading videos (25.7%).

CONCLUSION: More than half of the YouTube videos on gallstone disease are misleading. Credible videos uploaded by medical professionals and filtering by the staff of YouTube appear to be necessary.

Key words: YouTube; Gallstone disease; Gallstone; Gallbladder; Cholecystitis

Core tip: Many people now use the Internet for medical information. There have been many studies evaluating the available information on YouTube, which is one of the most popular sources of medical information. In this paper, we present the first report of an evaluation of YouTube videos on gallstone disease. More than half of the videos were misleading, and there was no correlation between video quality and the number of views or number of likes. Credible videos uploaded by medical professionals, and a filtering process appear to be necessary.

Lee JS, Seo HS, Hong TH. YouTube as a source of patient information on gallstone disease. *World J Gastroenterol* 2014; 20(14): 4066-4070 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4066.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4066

INTRODUCTION

In the past, most people sought medical information by consulting medical professionals; however, due to the development and spread of the Internet, many people now use online resources to access medical information[1]. One of the most popular sources of Internet-based medical information is YouTube (www.youtube.com). As opposed to search engines where a keyword brings up websites and images as well as videos, the search results of YouTube contain only video content. Unregistered users can watch videos, and registered users can upload an unlimited number of videos. The only limitation for viewers is that the videos considered offensive are available only to registered users 18 years or older[2]. Since Keelan et al. reported on the quality of YouTube videos regarding immunisation in 2007[3], many authors have analysed videos on topics such as prostate cancer, burns, and tonsillectomy[1,4-8]. As more medical professionals recognise the importance of YouTube as a source of medical information for the general public, the number of studies evaluating the quality of information found on YouTube is increasing[9].

Calculous gallbladder disease, or gallstone disease, is common. When asymptomatic gallstones are discovered, follow up is recommended, except in special circumstances such as porcelain gallbladder or large gallstones[10]. Symptomatic gallstone disease should be treated with cholecystectomy, preferably *via* laparoscopy[11]. The only medication known to dissolute gallstones in carefully selected patients is ursodeoxycholic acid[10]. When treated inappropriately, serious complications may occur. In this study, the authors aimed to evaluate the accuracy of medical information about gallstone disease available on YouTube. The second goal was to evaluate the response of the general population to the quality of videos. To the best of our knowledge, this is the first study to evaluate the quality of YouTube videos about gallstone disease.

MATERIALS AND METHODS

A YouTube search was performed on September 18, 2013. Keywords used in the search were gallbladder disease, gallstone disease, and gallstone treatment. One hundred videos were analysed for each keyword under the assumption that the user would not go beyond the first five pages of search results. The videos were sorted in order of relevance, which is the current YouTube default. Approval of the Institutional Review Board of the study institution was not required for this study. Three researchers (Lee JS, Seo HS, and an additional researcher, Kim KM) independently assessed the videos. All researchers had finished their respective residencies at tertiary centres and had sufficient experience in the diagnosis and management of calculous gallbladder disease. Characteristics such as name of video, source, length, number of views, number of "likes" and "dislikes", and days since upload were recorded. The upload source was categorised as physician or hospital (PH), medical website (MW) or TV channel, commercial website (CW), or civilian (CI). Videos with a primary content of "gallstone disease" were analysed. Videos not in English, videos with no audio, surgical videos, and videos aimed at professional medical personnel (such as medical school lectures) were excluded. A usefulness score was devised to assess the information in each video (Table 1). This score was used to categorise videos as very useful, useful, slightly useful, or not useful. Regardless of the usefulness score, videos with misleading content were categorised as "misleading" and were further categorised according to which treatment modality was advocated in the video. Disagreements between the researchers regarding the categorisation of a particular video were resolved by discussing the issue until a consensus was reached.

Table 1 Usefulness score criteria

Score criteria
Cause
Symptoms
Diagnosis
Treatment
Recovery

Not mentioned: 0; Mentioned briefly: 1; Mentioned in detail: 2. Total score: not useful (0), slightly useful (1-3), useful (4-7), very useful (8-10).

Differences between groups were compared with ANOVA, and Tukey's test was used for post hoc comparisons. Spearman's rank coefficient was used to analyse correlations. A weighted kappa score was calculated pairwise to evaluate the interobserver variability. Statistical analyses were performed with SPSS version 18 (SPSS Inc., Chicago, IL).

RESULTS

One hundred videos were analysed for each of the three keywords (gallbladder disease, gallstone disease, and gallstone treatment), and 135 duplicates were excluded. Of the remaining 165 videos, 34 were excluded based on the aforementioned exclusion criteria. A total of 131 videos were analysed. The mean length of the videos was 257 s, and each video was viewed an average of 14620 times.

Video demographics according to usefulness are shown in Table 2. More than half of the videos were misleading (74, 56.5%), while 36 of the videos (27.5%) were slightly useful, 15 (11.5%) were useful, and only three (2.3%) were deemed very useful. Three videos (2.3%) were categorised as not useful. The number of mean likes in the very useful group was 1.3 ± 1.5, which was significantly lower than the misleading group (17.2 ± 38.0, $P = 0.007$). The number of mean views in the very useful group was 756.3 ± 701.0, which was also significantly lower than in the misleading group (8910.7 ± 17094.7, $P = 0.001$). There were no other significant differences between groups regarding either the number of likes or the number of views. All three very useful videos were uploaded by a PH source. Among the 74 misleading

Table 2 Video demographics according to usefulness category

Video demographics	Usefulness of information					Total	*P* value
	Very useful	Useful	Slightly useful	Not useful	Misleading		
Videos, *n* (%)	3 (2.3)	15 (11.5)	36 (27.5)	3 (2.3)	74 (56.5)	131	-
Total length (h:min:s)	0:11:07	1:00:18	1:52:26	0:09:10	6:06:14	9:20:15	-
Mean length (h:min:s)	00:03:42 ± 00:01:17	00:04:05 ± 00:03:33	00:03:07 ± 00:03:20	00:03:03 ± 00:01:50	00:04:57 ± 00:05:10	00:04:17 ± 00:04:29	0.364
Mean "likes" (*n*)	1.3 ± 1.5	42.8 ± 145.8	10.6 ± 22.4	10.7 ± 18.5	17.2 ± 38.0	17.8 ± 57.7	0.007[1]
Mean "dislikes" (*n*)	0.3 ± 0.6	3.3 ± 11.6	0.9 ± 1.7	1.3 ± 2.3	2.9 ± 6.8	2.3 ± 6.5	0.158
Total views (*n*)	2269	1030071	205081	18476	659392	1915289	-
Mean views (*n*)	756.3 ± 701.0	68671.4 ± 248999.6	5696.7 ± 11330.7	6158.7 ± 10231.0	8910.7 ± 17094.7	14620.5 ± 85212.0	0.001[1]
Days since upload	518.7 ± 430.9	737.3 ± 480.4	706.2 ± 487.7	426.7 ± 331.9	518.3 ± 368.3	592.9 ± 423.7	0.126
Upload source, *n* (%)							
Physician	3 (100)	3 (20.0)	12 (33.3)	1 (33.3)	2 (2.7)	21 (16.0)	-
Website	0	12 (80.0)	18 (50.0)	1 (33.3)	0	31 (23.7)	-
Commercial	0	0	0	0	64 (86.5)	64 (48.9)	-
Civilian	0	0	6 (16.7)	1 (33.3)	8 (10.8)	15 (11.5)	-

[1]Comparison of very useful and misleading.

Table 3 Video demographics according to upload source

Video demographics	Upload source				Total	*P* value
	Physician or hospital	Medical website or TV channel	Commercial website	Civilian		
Videos, *n* (%)	21 (16.0)	31 (23.7)	64 (48.9)	15 (11.5)	131	-
Total length (h:min:s)	0:59:29	1:24:08	4:57:10	1:59:28	9:20:15	-
Mean length (h:min:s)	00:02:50 ± 00:01:31	00:02:43 ± 00:02:43	00:04:39 ± 00:05:06	00:07:58 ± 00:05:09	00:04:17 ± 00:04:29	0.009[1], 0.010[2]
Mean 'likes' (*n*)	10.9 ± 25.4	25.4 ± 101.9	17.6 ±40.3	12.7 ±15.1	17.8 ± 57.7	0.254
Mean 'dislikes' (*n*)	0.5 ± 1.8	2.1 ± 8.1	3.0 ± 7.2	2.3 ± 2.7	2.3 ± 6.5	0.083
Total views (*n*)	74549	1162278	581839	96623	1915289	-
Mean views (*n*)	3550.0 ± 7036.5	37492.8 ± 173179.9	9091.2 ± 18010.5	6441.5 ± 8558.2	14620.5 ± 85212.0	0.168
Usefulness information *n* (%)						
Very useful	3 (14.3)	0	0	0	3 (2.3)	-
Useful	3 (14.3)	12 (38.7)	0	0	15 (11.5)	-
Slightly useful	12 (57.1)	18 (58.1)	0	6 (40.0)	36 (27.5)	-
Not useful	1 (4.8)	1 (3.2)	0	1 (6.7)	3 (2.3)	-
Misleading	2 (9.5)	0	64 (100)	8 (53.3)	74 (56.5)	-

[1]Civilian *vs* website; [2]Civilian *vs* physician.

videos, 64 (86.5%) were uploaded by CW, eight (10.8%) were uploaded by CI, and two (2.7%) were uploaded by PH. A Spearman's rank correlation analysis showed no correlation between the usefulness category and number of views (r = 0.065, P = 0.464), number of likes (r = -0.038, P = 0.663), or video length (r = -0.151, P = 0.086).

Video demographics according to the upload source are shown in Table 3. The highest number of videos was uploaded by a CW (64, 48.9%). Thirty-one videos (23.7%) were uploaded by MW, 21 videos (16.0%) were uploaded by PH, and 15 (11.5%) were uploaded by CI. The mean length of the CI videos was significantly longer (7.58 ± 5.09) compared with the PH videos (2.50 ± 1.31, P = 0.01) and the MW videos (2.43 ± 2.43, P = 0.009). There were no significant differences in the mean length between the other groups. There were no differences in the mean number of likes received or the mean number of views.

Table 4 shows various treatment methods advocated by misleading videos. The highest number of these videos advocated the "gallstone flush" (39, 25.7%). Twenty-four videos (32.4%) advocated medication that can dissolve gallstones, and six videos (8.1%) advocated herbal treatment.

The interobserver variability was calculated as a weighted kappa score of 0.94 between Lee JL and Seo HS, 0.84 between Seo HS and Kim KM, and 0.80 between Lee JL and Kim KM.

DISCUSSION

This study evaluated the content quality of YouTube videos regarding gallstone disease. Out of 131 videos, 74 (56.5%) videos were misleading. This percentage is disturbingly high compared with that of previous studies evaluating video content in other fields[1,4,6]. Recent advances in imaging technologies have led to an increase in the diagnosis of asymptomatic gallstones. In contrast to appendicitis patients or hernia patients, patients with asymptomatic gallstones have more time to seek medical information about their condition. In turn, many people appear to be targeting these patients with the aim of profiting.

Table 4 Methods advocated by misleading videos

Misleading videos	*n* (%)
Gallstone flush	39 (52.7)
Medication	24 (32.4)
Herbal treatment	6 (8.1)
Other	5 (6.8)
Total	74 (100)

Notably, videos uploaded by commercial websites were even less credible than videos uploaded by civilians. It appears that commercial entities did not perform any research before uploading these videos. The high percentage of misleading videos reflects the abundance of commercial products advertising treatment of gallstones without surgery. The "gallstone flush" is by far the most popular method[12]. Many commercial websites advertise books that carry specific guidelines for the gallstone flush. In this method, a patient with gallstones drinks olive oil and lemon juice following a specific protocol. Three to five days later, the patient passes several "gallstones". These "stones" have been found to be simply the product of the mixture of oil and lemon juice[13]. There are also various medications that allegedly detoxify the gallbladder and remove the gallstones[14,15].

Not only are these videos misleading, they are potentially fatal. Even more concerning than these commercial websites are the two videos uploaded by physicians with misleading content. In one video, a medical doctor states that the treatment of choice for symptomatic gallstones is a low fat diet[16]. In the other video, a medical doctor claims that drinking herbal tea can dissolve gallstones[17]. These two videos may do even more harm than the commercial videos.

With these facts in mind, the most important issue is that the general population tends to view the misleading videos more than the credible videos. Biggs *et al*[1] suggest that this is because useful videos tended to be longer than misleading videos. In the present study, all of the videos were approximately the same length, yet the videos that were deemed very useful had significantly fewer views and likes than the misleading videos. In a study performed by Butler *et al*[4], the usefulness score and the number of views were only weakly correlated. The present study found no correlation between the usefulness and the number of views or number of likes.

Interestingly, while the weighted kappa score was 0.94 between the two researchers who received training at the same centre, the score was 0.84 and 0.80 between these two researchers and another researcher who had received training at a different centre. Although the range of kappa scores demonstrates significant interobserver agreement, it also demonstrates that the training of the researcher influenced their assessment of the videos.

This study has several limitations. First, a subjective score criteria was used to evaluate the videos, as there are as of yet no validated tools for assessing video data. Second, the study results may change according to the keywords used in the search. This study used "gallbladder disease", "gallstone disease", and "gallstone treatment" under the assumption that these are the keywords a layperson would choose rather than "cholecystitis" or "cholelithiasis". This may not always be the case. Third, the evaluated videos were sorted by relevance, which is the YouTube default. This relevance may have been affected by advertisements, and the results may be different when sorted with another standard. Lastly, these results demonstrate the quality of information at one point in time, and results may change with time as videos are added or removed.

In summary, more than half of the YouTube videos regarding gallstone disease are misleading and present a risk of harmful consequences. Credible videos with accurate information need to be uploaded by medical professionals and medical institutions. Active filtering by the managing staff of YouTube may also be necessary.

COMMENTS

Background

Due to the development and spread of the Internet, many people use the Internet as a source of medical information. One of the most popular of these sources is YouTube.

Research frontiers

As more medical professionals recognise the importance of YouTube as a source of medical information for the general public, studies have increasingly evaluated the quality of YouTube videos on a wide range of topics such as prostate cancer, burns, and tonsillectomy.

Innovations and breakthroughs

This was the first study to evaluate YouTube videos on gallstone disease. The results showed that more than half of the videos were misleading and that the quality of the videos did not correlate with the number of likes or number of views.

Applications

Gallstone disease is very common, and the public should be informed that widely advertised methods such as the "gallstone flush" are ineffective and may delay appropriate treatment. A method for filtering misleading information is necessary.

Terminology

When viewing a video on YouTube (www.youtube.com), the viewer can click on an icon showing a thumbs-up gesture. This is known as a "like" and generally shows that the viewer is satisfied with the content of the video. The number of times viewers have clicked "like", as well as the total number of views for each video is shown.

Peer review

The current study is a review of YouTube videos on the topic of gallbladder disease. The findings show that a significant portion of these videos are misleading to viewers and that low quality videos are more frequently distributed by commercial entities. The study provides an interesting topic with clinical significance due to an increasing number of online resources utilised by patients.

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RESEARCH REPORT

Efficacy and tolerability of low-dose interferon-α in hemodialysis patients with chronic hepatitis C virus infection

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Abstract

AIM: To evaluate the efficacy and tolerability of low-dose standard or pegylated interferon (PEG-IFN) in hepatitis C virus (HCV)-positive hemodialysis patients.

METHODS: In total, 19 patients were enrolled in this study, of which 12 received PEG-IFNα-2a 67.5 μg 1 time/wk (Group 1) and 7 received standard interferon α-2b subcutaneously 1.5 × 10^6 U 3 times/wk (Group 2). The treatment durations were 48 wk for patients infected with HCV genotype 1 and 24 wk for patients infected with HCV genotype 2/3. All patients were prospectively followed after the completion of therapy. The efficacy and tolerability of the treatment were evaluated based on the sustained virological response (SVR) and treatment-related drop-out rate.

RESULTS: In Group 1, 11 of the 12 patients completed the treatment. Early virological response (EVR) and sustained virological response (SVR) rates were 83.3% and 91.7%, respectively. One patient withdrew from treatment due to an adverse event (leukopenia). The drop-out rate was 8.3% in this group. In Group 2, 5 of the 7 patients completed the treatment with an EVR and SVR of 85.7% and 71.4%, respectively. Two patients withdrew due to treatment-related adverse events (nausea and depression). In this group, the drop-out rate was 28.6%. In total, 16 of the patients attained EVR, and 15 of them completed the treatment. The SVR rate for the patients who attained EVR was 93.7%. Anemia was the most frequent side effect and was observed in 10/19 patients (55.5%), but could be effectively managed with erythropoietin.

CONCLUSION: Low-dose interferon monotherapy, either with PEG-IFNα-2a or standard interferon α-2b, is an effective treatment option for hemodialysis patients with chronic hepatitis C.

Key words: Chronic hepatitis C; End-stage renal disease; Hemodialysis; Hepatitis C virus; Peginterferon

Core tip: The most appropriate treatment for hepatitis C virus (HCV)-positive hemodialysis patients is unknown, and the available treatments have only been assessed in a limited manner. Therefore, this study evaluated the efficacy and tolerability of treatment with low-dose standard or pegylated interferon (PEG-IFN) in HCV-positive hemodialysis patients. The results of the study indicated that low-dose interferon monotherapy, either PEG-IFNα-2a or standard interferon α-2b, is an effective treatment option for HCV-positive hemodialysis patients. Anemia was the most frequently encountered adverse event, but this could be managed with erythropoietin. These results provide important information for clinicians faced with these treatment decisions.

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INTRODUCTION

End-stage renal disease (ESRD) is a significant problem worldwide. In China, more than 65000 ESRD patients have received maintenance hemodialysis (HD)[1]. These patients have a higher rate of chronic hepatitis C virus (HCV) infection compared with the overall population[2], with an estimated prevalence and incidence of 3%-80% and 0.33%-2.59%, respectively[2-5]. Moreover, chronic HCV infection might ultimately lead to severe liver lesions, cirrhosis, and hepatocellular cancer, further increasing the morbidity and mortality of ESRD patients[6,7].

Interferon combined with oral ribavirin is the standard treatment for chronic HCV[8]. However, the use of ribavirin is currently contraindicated in dialysis patients[9,10], as the drug is not removed during conventional dialysis and its accumulation causes dose-dependent hemolytic anemia[11,12]. Interferon monotherapy is therefore recommended for the treatment of dialysis patients with chronic hepatitis C[13]. However, experience with monotherapy in ESRD patients is still limited.

The available data suggest that the rate of adverse events and drop-outs in this patient population is high[14-16]. We hypothesized that a lower dose of interferon monotherapy may decrease the incidence of adverse events and drop-outs, thus improving the likelihood of completing treatment. Therefore, the objective of this study was to evaluate the relative efficacy and tolerability of treatments consisting of either 1.5×10^6 U 3 times/wk of standard interferon or 67.5 μg/wk of pegylated interferon (PEG-IFN) α-2a (40 kDa). The treatments lasted for 48 wk in ESRD patients infected with HCV genotype 1 and for 24 wk in ESRD patients infected with HCV genotype 2/3.

MATERIALS AND METHODS

Patients

A total of 19 patients (11 male and 8 female) with a mean age of 37.7 years (range: 27-58 years) were enrolled in this study. The subjects had been undergoing dialysis for an average of 7.3 ± 4.5 (range: 2-16) years. All patients were on a maintenance HD program for ESRD, with 4-h dialysis sessions, 3 times/wk, using high-flux synthetic membranes, and bicarbonate dialysate. All patients were positive for HCV antibodies and had demonstrated detectable HCV RNA by polymerase chain reaction for at least 3 mo. The patients' HCV genotypes were confirmed before treatment. All the treated patients met the criteria for treatment with PEG-IFNα-2a or standard interferon α-2b.

Treatment and follow-up

All patients were recommended to receive intramuscular injections of 67.5 μg PEG-IFNα-2a once/wk after their HD session. For economic reasons, only 12 patients (8 male and 4 female; mean age: 39.4 ± 9.4 years) received this treatment (Group 1). The other 7 patients (3 male and 4 female; mean age: 34.7 ± 6.5 years) received subcutaneous injections of standard interferon α-2b, 1.5×10^6 U 3 times/wk, which was also administered after each HD session (Group 2). The treatment durations were 48 wk for patients infected with HCV genotype 1 and 24 wks for patients infected with HCV genotypes 2/3/6.

All treated patients were prospectively followed until week 96, which corresponded to 48 wk after the completion of the treatment, to assess the long-term efficacy and tolerability of the lower-dose interferon monotherapy. Blood samples were collected weekly to determine blood cell counts, and monthly to determine liver function. The presence of HCV RNA was assessed prior to treatment and then every 3 mo after treatment. Early virological response (EVR), defined as a ⩾ 2 log-fold decrease in HCV RNA from baseline, was evaluated after 12 wk of treatment. The efficacy of the treatment was determined by the achieved end treatment virological response (ETR), defined as the absence of detectable serum levels of HCV RNA at week 48, and sustained virological response (SVR), defined as the absence of detectable serum levels of HCV RNA at week 72. Tolerability was evaluated by assessing the drop-out rates and serious adverse events.

Statistical analysis

The data were analyzed using SPSS software (Windows version 15.0; SPSS, Chicago, IL, United States) and are presented as the means ± SD for parameter variables.

RESULTS

The baseline values of the patients are shown in Table 1, and the outcomes are presented in Table 2.

Efficacy

In Group 1, 11 of the 12 patients completed the treatment, with 1 patient withdrawing from treatment due to an adverse event. In this group, EVR was observed in 10 patients at week 12, and ETR was achieved in 11 patients. The 11 patients who attained ETR also achieved a SVR.

In Group 2, EVR was observed in 6 patients. In total, 5 patients completed the treatment, and ETR and SVR were recorded in all of these patients. In Group 2, 2 patients withdrew from treatment.

The HCV genotype was type 1 in 16 patients, with 13 patients achieving a SVR, yielding a genotype 1 SVR of 81.3%. The 3 patients with HCV genotype 2 all attained a SVR. In total, 16 of the patients attained EVR, and 15 of them completed the treatment. The SVR rate for the patients who attained EVR was 93.7%.

Table 1 Patient baseline characteristics

Case	Sex	Age (yr)	Weight (kg)	HD duration (yr)	Cirrhosis	Genotype	HCV RNA (IU/mL)
1	M	52	62	5	N	1b	4805000
2	F	28	40	10	N	1b	3150000
3	M	28	69	14	N	1b	15840
4	M	38	52	6	N	1b	3600000
5	F	38	54	16	N	1b	3786000
6	F	36	47	2	N	1b	167000
7	F	47	48	14	N	1b	663000
8	M	40	59	9	N	1b	356000
9	M	39	63	13	N	1b	156000
10	M	41	67	2	N	2a	15580
11	F	58	50	6	N	2a	9590
12	M	28	55	2	N	1b	209200
13	M	41	63	3	N	1b	39460
14	M	33	59	6	N	1b	143000
15	F	32	52	7	N	2a	446400
16	M	36	67	6	N	1b	456000
17	F	27	51	4	N	1b	14500
18	F	29	49	4	N	1b	12200
19	M	45	62	11	Y	1b	266000

HCV: Hepatitis C virus; HD: Hemodialysis; F: Female; M: Male.

Table 2 Patient treatment results

Case	IFN-α type	HCV RNA (IU/mL)				
		12 wk	48 wk	72 wk	96 wk	144 wk
1	PegIFNα-2a	405100	Positive	Positive	Positive	Positive
2	PegIFNα-2a	Negative	Negative	Negative		
3	PegIFNα-2a	Negative	Negative	Negative	Negative	Negative
4	PegIFNα-2a	Negative	Negative	Negative	Negative	Negative
5	PegIFNα-2a	Negative	Negative	Negative	Negative	
6	PegIFNα-2a	Negative	Negative	Negative	Negative	
7	PegIFNα-2a	2380	Negative	Negative	Negative	
8	PegIFNα-2a	Negative	Negative	Negative	Negative	
9	PegIFNα-2a	Negative	Negative	Negative		
10	PegIFNα-2a	Negative	Negative	Negative	Negative	
11	PegIFNα-2a	Negative	Negative	Negative	Negative	
12	PegIFNα-2a	Negative	Negative	Negative	Negative	
13	IFNα-2b	Negative	Negative	Negative		
14	IFNα-2b	Negative	Negative	Negative	Negative	
15	IFNα-2b	Negative	Negative	Negative	Negative	
16	IFNα-2b	256000	Positive	Positive		
17	IFNα-2b	Negative	Negative	Negative		
18	IFNα-2b	Negative	Negative	Negative		
19	IFNα-2b	Negative	Positive	Positive		

IFN: Interferon; PEG-IFN: Pegylated interferon; HCV: Hepatitis C virus.

All patients were followed post-treatment, and those who achieved a SVR had undetectable HCV RNA levels at week 96. Notably, 2 patients continued to have undetectable levels of HCV RNA at 144 wk, which was the last follow-up prior to the writing of this manuscript.

Tolerability

In Group 1, 1 patient withdrew from treatment due to significant leukopenia (8.2% drop-out rate). Two patients discontinued the standard interferon α-2b treatment (Group 2) due to treatment-related adverse events; one patient developed nausea (which required treatment cessation after 4 wk of therapy) and the other patient withdrew from treatment at week 20 because of depression (28.6% drop-out rate).

Anemia was one of the treatment-related adverse events observed in both groups. This adverse event was observed in 3 of the 7 (42.8%) patients in the standard interferon group and in 7 of the 12 (58.3%) patients treated with PEG-IFN. In these patients, the erythropoietin dose had to be increased to improve hemoglobin levels. However, the treatment protocol did not have to be discontinued in any patients due to anemia.

Other adverse events included influenza-like symptoms, nausea, poor appetite, leucopenia, and thrombocytopenia, as has been reported in other studies.

DISCUSSION

Interferon monotherapy trials involving small numbers of dialysis patients infected with chronic hepatitis C have previously been reported. In addition, 3 meta-analysis studies have shown that the SVR of ESRD patients infected with chronic hepatitis C and treated with standard IFN monotherapy was approximately 31%-41%[17-19]. However, the corresponding treatment-related withdrawal rate ranged from 20% to 30%[16,17,20]. Similarly, studies have also shown that the SVR and treatment-related withdrawal rates in patients receiving PEG-IFN were 31%-37% and 23%-28%, respectively[17,20,21]. Based on these studies, treatment of patients with either standard interferon or PEG-IFN had similar efficacy and tolerability.

In our study, 7 hemodialysis patients were treated with standard interferon α-2b for 48 wk. Six patients (85.7%) achieved EVR by week 12. In total, 2 patients discontinued treatment because of adverse events (1 due to nausea at week 4 and 1 due to depression at week 20), with both having a high viral load at baseline. At the end of week 72, the SVR rate was 71.4% (5/7 patients), with a drop-out rate of 28.6%. The drop-out rate was similar to that previously described in the meta-analyses. The 11 patients treated with PEG-IFNα-2a completed the treatment, and the EVR and SVR were 83.3% and 91.7%, respectively. For the patients who achieved a SVR, this subsequently remained stable.

Contrary to the concept that HCV genotype 1 is associated with a poor SVR rate in response to interferon monotherapy in HCV patients with normal renal function[22,23] our results showed a good SVR rate in HD patients. Although the 3 patients who dropped out all had genotype 1, the SVR rate for the remaining 16 patients with genotype 1 HCV was 81.3%, higher than that reported for HCV patients with normal renal function. Notably, all of the patients who dropped out had a very high HCV RNA load, which is considered as a negative predictor of a SVR[22,23].

In our study, 16 patients achieved an EVR, and 15 of them achieved a SVR. Therefore, patients with an EVR might be encouraged to continue therapy, as EVR appears to be a good predictor of a SVR. In this respect, our study is in agreement with the current literature.

The overall SVR for the 19 patients in this study was 84.2%, higher than that for interferon therapy in HCV patients with normal renal function. This result may be explained by the fact that the patients in the present study were younger (mean age 37.7 years), had a lower mean weight (55.5 ± 8.7; range 47-67 kg), and had a lower prevalence of cirrhosis (1/19). Being of Asian descent has also been reported to be an independent predictor of SVR in some studies[24]. Some studies have also suggested that hepatitis C dialysis patients usually have a lower viral load[25] and increased endogenous interferon release from circulating white blood cells[26].

Anemia was the most frequently encountered adverse event in both of the present study groups. However, in most cases, it was adequately managed with erythropoietin. The other adverse events observed included fatigue, headache, body ache, fever, nausea, reduced appetite, leucopenia, and thrombocytopenia. Regardless, low-dose interferon monotherapy did not result in a drop-out rate similar to what was expected for standard interferon monotherapy based on the results from non-HD patients.

This study has 2 major limitations: a small sample size and a lack of randomization for the 2 treatment groups, with the latter resulting in the lack of standard controls. Thus, these results may not be broadly extrapolated to the wider HD population.

In conclusion, either PEG-IFNα-2a or standard interferon α-2b monotherapy is an effective treatment option for HD patients with chronic hepatitis C. Anemia was the most frequently encountered adverse event in most cases and was effectively managed with erythropoietin. However, because of the small number of patients in this study, the conclusions of this study cannot be broadly generalized. Studies with low-dose interferon monotherapy are ongoing, and further long-term, large, randomized multicenter studies with larger patients and control populations are needed.

COMMENTS

Background

Hepatitis C virus (HCV) infection remains frequent in hemodialysis patients worldwide. However, the most appropriate treatment for HCV-positive hemodialysis patients is unknown.

Research frontiers

The present study was carried out in hemodialysis patients with chronic hepatitis C. Nineteen hemodialysis patients were included in this study.

Innovations and breakthroughs

The overall sustained virological response (SVR) for the 19 patients in this study was 84.2%, higher than that for interferon therapy in HCV patients with normal renal function.

Applications

This study showed that low-dose interferon monotherapy, either with pegylated interferon α-2a or standard interferon α-2b, is an effective treatment for hemodialysis patients with chronic hepatitis C.

Terminology

SVR indicates sustained virological response, which means sustained (more than 24 wk after treatment) viral clearance from the infected host.

Peer review

The authors investigated the effects and tolerability of low-dose interferon monotherapy for treatment of HCV-positive hemodialysis. The result showed either PEG-IFNα-2a or standard interferon α-2b monotherapy as an effective treatment option for HD patients with chronic hepatitis C.

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Clinical significance of nerve growth factor and tropomyosin-receptor-kinase signaling pathway in intrahepatic cholangiocarcinoma

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Abstract

AIM: To investigate the correlation between nerve growth factor-tropomyosin-receptor-kinase (NGF-TrkA) signaling pathway and prognosis in intrahepatic cholangiocarcinoma (IHCC).

METHODS: NGF and TrkA expression in 83 samples of IHCC was assessed by immunohistochemistry. Correlations between NGF-TrkA expression and clinicopathological features were analyzed by χ^2 test. Moreover, we evaluated the association between NGF-TrkA and overall survival by univariate and multivariate analysis. With experiments *in vitro*, we investigated the crucial role of NGF-TrkA on proliferation and invasion of IHCC cells with recombinant NGF-β stimulation.

RESULTS: We found that NGF and TrkA expression was significantly related with differentiation (P = 0.024) and intraneural invasion (P = 0.003), respectively. Additionally, double higher expression of NGF and TrkA was identified as an independent prognostic factor in IHCC (P = 0.003). Moreover, we demonstrated that NGF-TrkA signaling pathway can promote IHCC proliferation and invasion.

CONCLUSION: NGF-TrkA double higher expression is an independent prognostic factor in IHCC. NGF-TrkA pathway can promote IHCC progression, indicating that NGF-TrkA may become a potential drug target.

Key words: Nerve growth factor; Tropomyosin-receptor-kinase; Prognosis; Intrahepatic cholangiocarcinoma; Progression

Core tip: For the first time, we systemically investigated nerve growth factor (NGF) and tropomyosin-receptor-kinase (TrkA) expression in 83 intrahepatic cholangiocarcinoma(IHCC) samples by immunohistochemistry, and then analyzed the expression relationship with clinicopathological features, which resulted in finding that NGF and TrkA were significantly associated with differentiation (P = 0.024) and intraneural invasion (P = 0.003) respectively. Moreover, we found that NGF and TrkA double higher expression had poorer prognosis than others. NGF-TrkA double higher expression was further confirmed as an independent prognostic factor by multivariate analysis. With function assays we demonstrated that NGF-TrkA signaling pathway played a crucial role in cholangiocarcinoma proliferation and invasion, indicating NGF-TrkA pathway could be a promising potential drug target of intrahepatic cholangiocarcinoma.

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INTRODUCTION

Cholangiocarcinoma (CCA) is highly malignant tumor that arises from biliary tract. The 7th union for international cancer control/american joint committee on cancer (UICC/AJCC) divided CCA into the intrahepatic, perihilar and distant cholangiocarcinoma according to their location and different clinical features. Incidence of CCA just accounts for 3% of gastrointestinal cancer[1,2], but the morbidity and mortality of cholangiocarcinoma, especially intrahepatic cholangiocarcinoma (IHCC), are increasing worldwide[3]. The IHCC is featured of silent clinical signatures, early regional invasiveness, distant metastasis and poor prognosis[4-6]. Currently, radical resection of tumor and involved liver is the only curative treatment for IHCC. However, when patients are hospitalized because of jaundice or pain, it is usually too late for surgery owing to the early invasiveness and metastasis, which make the resectability rate quite low and variable (18%-70%)[7]. Hence, both effective pre- and post-operational biomarkers are urgently needed for early and individualized treatment. Unfortunately, the process of researching mechanisms and biomarkers of IHCC was very slow, though some breakthroughs were realized in molecular analysis and classification[8,9].

Nerve growth factor (NGF) was the first discovered critical member in the neurotrophin polypeptide family[10], which is comprised of brain-derived neurotrophic factor, neurotrophins NT-3, NT-4/5, NT-6, and NT-7[11]. NGF functions as a signaling molecule by binding with two known receptor: the common p75 neurotrophin receptor (p75NTR) which binds all of the neurotrophins with almost equal affinity, and specific tyrosine kinase receptors called tropomyosin related kinases (Trks) which binds neurotrophin specially[12,13]. Dysregulation of NGF was found in many kinds of tumors including neuronal tumors and non-neuronal tumors like prostate, lung, and breast cancers[14]. Moreover, rearrangements or aberrant expression of Trk genes were found in a variety of other cancers such as papillary thyroid carcinomas, secretory breast cancers, pediatric sarcomas and leukemias[15]. For example, tropomyosin-receptor-kinase receptor, the only Trk receptor which can bind with NGF, is demonstrated to be correlated with breast cancers, thyroid carcinomas and neuroblastomas[16-19]. NGF has been proved to activate Raf-MAPK signaling pathway, which is well acknowledged to be related with carcinogenesis[20]. For a long period of time, NGF and its receptors are considered as a potential molecular target of tumorgenesis. In regard to CCA, NGF itself was indicated to promote CCA cell line progression in study *in vitro*[21]. In perihilar CCA, NGF-β was proved to be associated with lymph node metastasis and nerve infiltration[22]. However, the relation between NGF and IHCC has not been reported in clinical study, and no article has proved the role of NGF-tropomyosin-receptor-kinase (NGF-TrkA) signaling in IHCC.

In our study, we investigated expression of NGF and TrkA in 83 samples of IHCC by IHC, and further explored the relationship of NGF/TrkA expression with clinicopathologic parameters and overall survival rates. To explain why NGF-TrkA signaling pathway was associated with poor prognosis of IHCC, we used two IHCC cell lines and performed tumor function assays *in vitro,* including proliferation and invasion assay.

MATERIALS AND METHODS

Patients and follow-up

All the 83 IHCC samples were obtained from the tumor resection between 2002 and 2010. All the samples were obtained from the Department of Pathology of Qilu Hospital and Yishui Central Hospital, Shandong Province. The diagnosis was confirmed by the routine pathology and histopathological samples were reviewed by a senior pathologist to select suitable areas for immunohistochemical detection. The overall survival time was calculated from the operation to the date of death or censored at the date of the last follow-up examination. Clinical data, including age, gender and other clinicopahtologic features were abstracted from the patients' medical records. Pathologic tumor-node-metastasis (pTNM) staging was based on the 7th staging classification of International Union Against Cancer (2009).

This study was approved of the Institutional Clinical Ethics Review Board with prior patient consents. The clinical follow-up was at least 3 mo after surgery, with the median follow-up time 25.1 mo (from 3 to 96 mo). Criteria of the validation cohort included: (1) available formalin-fixed tumor tissues; (2) available clinical follow-up data and complete medical records; and (3) no history of previous anticancer therapy and other malignancies.

Cell culture and reagents

Human intrahepatic cholangiocarcinoma cell lines RBE and QBC939 were bought from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell line HUCCT-1 was purchased from RIKEN Bioresourse Center (Saitama, Japan). All the cells were cultured in the RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco) and 1% ampicillin/streptomycin in 5% CO_2 resuscitation.

Recombinant human β-NGF was obtained from Sino Biological Inc. (Beijing, China). Matrigel pre-coated transwell was from BD Biosciences. All other reagents were bought from Sigma Company. TrkA antibody was purchased from Santa Cruz (Cat No. sc-118), NGF antibody was from Abcam Company (Cat No. ab52918), phos-

phor-TrkA-Y490, phosphor-AKT-S473 and phosphor-ERK-T202/204 antibodies were obtained from Cellsignaling Technology.

Immunohistochemistry and evaluation

Streptavidin peroxidase complex method was used for immunohistochemical (IHC) staining referring to previous study[14,23,24]. Adhesive-coated slide was used to transfer tissue microarray sections, and then samples were de-paraffinized and rehydrated with xylene and graded alcohol. Slides were then incubated in 3% hydrogen peroxide for 60 min to quench endogenous activity, and then immersed in citrate buffer (pH = 6.0) for antigen retrieval. Microwave oven was used to heat the buffer for 15 min for satisfied antigen retrieval. The sections were then blocked with 1% BSA in PBS containing 10% normal serum for 30 min at 37 ℃. Slides were incubated in the corresponding primary antibodies (at the dilution of 1:50) overnight at 4 ℃. Secondary antibodies labeled with streptoavidin-biotin-peroxidase reagent were used after removal of primary antibody and PBS washing. For visualization, slides were incubated in the 3,3'-diaminobenzidine solution until desired staining was approached. Lastly, slides were counterstained with hematoxylin and mounted.

The score of IHC staining was based on the multiply of staining intensity and area. The staining intensity of all tested proteins was scored as negative (0), weak (1), moderate (2) and strong (3), and scores of stained area was defined as follows: 1, < 10% of cells were positive; 2, 10%-50% of cells were positive; 3, > 50% of cells were positive. The mean score of NGF and TrkA was 3.4 and 2.5 respectively. The samples were divided into higher and lower groups according to the average score, namely the cut-off.

Immunoblotting

Cells were lysed first after treatment on ice with the lysis buffer(1% NP-40, 10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L sodium vanadate, 10 μg of leupeptin, 1 μg of aprotinin, 1 μg of pepstatin, 1 μg of antipain, and 30 μg of phenylmethylsulfonyl fluoride per mL). Cells were centrifuged at 10000 *g* at 4 ℃ for 15 min after scraping and the superior was added with loading buffer with equal volume. Protein concentration was tested by a BCA kit (Merck Company) and equal quality of protein was added to run SDS-PAGE gel. Protein in the gel was subsequently transferred into a PVDF membrane(PALL, United States) and incubated in 5% skimmed milk to block unspecific binding and then incubated in primary antibody with dilution at 1:1000 in 4 ℃ overnight. Corresponding secondary antibody was added after washing the membrane 3 times and protein was visualized by adding ECL (Millipore Company, United States).

Proliferation assay

MTT assay was used to measure the proliferative activity of CCA cells. In brief, RBE cells were split into a 96-well plate with density at 5000 cells per well and then starved in serum-free medium overnight before stimulation. 100 ng/mL NGF-β was used to stimulate cells for 48 h. After stimulation, 10 uL MTT at 10 mg/mL concentration was added into medium and incubated for 4-6 h. Subsequently, medium was decanted and crystals were dissolved by 100 μL DMSO and incubated for 15 min for complete solution. Absorbance at 490 nm was read by a microplate reader and all data were standardized by compare with base line, and three independent experiments were performed to confirm results.

Invasion assay

Cell invasive activity was evaluated by transwell assay with matrigel-precoated transwell chambers (BD Company, United States). RBE cells were split into the chambers and incubated in serum-free medium for 6 h before 100 ng/mL NGF stimulation. After starvation, medium was changed to RPMI1640 with 1% fetal bovine serum, and NGF was added into lower filter. After 24 h, cells on upper filter surface were removed using a cotton swab and invasive cells were stained by crystal violet. Cell numbers were counted at × 200 magnification of at least five random visual fields and three independent experiments were performed to confirm results.

siRNA transfections

Both of the oligo siRNA and scramble RNA of NGF (sc-43970) and TrkA (sc-36726) were purchased from Santa Cruz Biotechnology. Growing cells were transiently transfected with either 50 μmol/L of siRNA or the scrambled siRNA control with Lipofectamine 2000™ (Invitrogen) according to the manual. Forty-eight hours following transfection, transfected cells were scraped and immunoblotting was used to confirm the successful knock down.

Statistical analysis

All the statistical analyses were carried out with SPSS 17.0 software (IBM company, United States). The association between protein expression and clinicopathologic parameters was evaluated by χ^2 test. Kaplan-Meier method was used to analyze correlation between survival rate and NGF-TrkA expression, and Cox Regression Model was used for multivariate analysis to determine the independent prognostic factors. P values < 0.05 was considered to be significant. The statistical comparisons between control and tested group were made with the Student t tests (The software Graph Pad Prism 5 was also used for statistical analysis).

RESULTS

NGF and TrkA expression in IHCC

As a secreted growth factor, NGF was mostly found in cytoplasm (Figure 1A and B), and TrkA was found on membrane or in cytoplasm (Figure 1C and D). According to the criteria described before, expressions of NGF

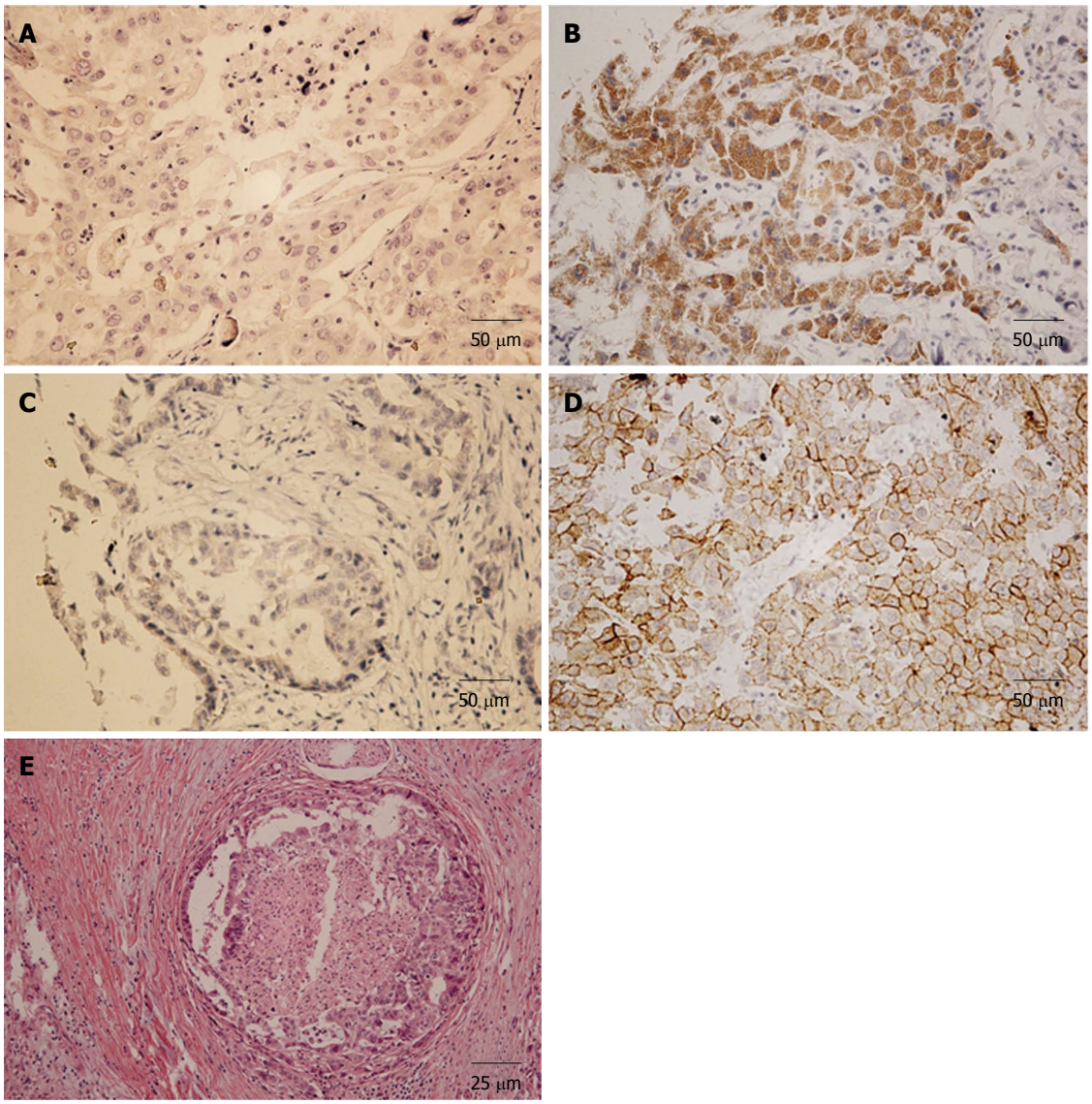

Figure 1 Nerve growth factor and tropomyosin-receptor-kinase expression in intrahepatic cholangiocarcinoma. Representative figures showing lower NGF expression (A), higher NGF expression (B), lower TrkA expression (C), higher TrkA expression (D), intraneural invasion by CCA cells (E). NGF: Nerve growth factor; CCA: Cholangiocarcinoma; TrkA: Tropomyosin-receptor-kinase.

and TrkA were divided into higher and lower expressive groups by the average cut-off. NGF was observed higher expressed in 27.7% (23/83) IHCC samples while TrkA was overexpressed in 20.5% (17/83) samples. Double NGF and TrkA higher expression was defined as both NGF and TrkA had higher score than corresponding cut-off. The percent of double NGF and TrkA higher expression was 15.6% (13/83).

Correlation between NGF, TrkA and clinicopathologic parameters

To further investigate the clinical and pathological importance of NGF and TrkA receptor, correlations between NGF, TrkA and clinicopathologic features were analyzed by Chi-Square method (Table 1). Well differentiation has more cases with higher NGF expression and poor differentiation has less cases with higher expression ($P = 0.024$) (Table 1). Moreover, TrkA expression was closely related to intraneural invasion ($P = 0.003$) (Figure 1E), which indicated that intraneural invasion may be resulted from the TrkA signaling pathway ectopic activation. This kind of TrkA activation may be caused by TrkA overexpression and consistent NGF stimulation, which probably come from the neuron or cancer cell autocrine.

Correlation between NGF, TrkA and overall survival rates

In univariate analysis, T stage ($P = 0.002$), N stage ($P = 0.004$) and TNM stage ($P = 0.004$) were significantly associated with the 5-year overall survival rate (Table 2). Unexpectedly, expression of NGF and TrkA alone had no significant influence on survival rate ($P = 0.201$ and 0.483 respectively) (Figure 2A and B). However, considering that NGF and TrkA may affect cell survival as a network, we further divided IHCC into group of NGF/TrkA higher-expression (both NGF and TrkA have higher score than the cut-off) and group of others (including

Table 1 Correlations between nerve growth factor/tropomyosin-receptor-kinase expression and clinicopathologic parameters

Clinicopathologic parameters		*n*	NGF		*P* value[1]	TrkA		*P* value[1]
			low	high		low	high	
Age	< 65 yr	62	44	18	0.640	49	13	0.850
	⩾ 65 yr	21	16	5		13	4	
Gender	Male	44	30	14	0.373	32	12	0.099
	Female	39	30	9		34	5	
Tumor size	< 5 cm	37	24	13	0.220	28	9	0.438
	⩾ 5 cm	46	36	10		38	8	
Differentiation	Well	19	9	10	0.024	20	5	0.228
	Moderately	39	32	7		34	5	
	Poorly	25	19	6		12	7	
T stage	T1	40	29	11	0.200	33	7	0.428
	T2	20	17	3		16	4	
	T3	23	14	9		17	6	
	T4	0	0	0		0	0	
N stage	N0	57	40	17	0.520	46	11	0.695
	N1	26	20	6		20	6	
M stage	M0	79	57	22	0.900	63	16	0.823
	M1	4	3	1		3	1	
TNM stage	Ⅰ	31	22	9	0.948	26	5	0.470
	Ⅱ	10	9	1		8	2	
	Ⅲ	16	9	7		12	4	
	Ⅳa	22	17	5		17	5	
	Ⅳb	4	3	1		3	1	
Satellites	N	63	45	18	0.754	51	12	0.572
	P	20	15	5		15	5	
Macrovascular invasion	N	80	57	23	0.158	63	17	0.236
	P	3	3	0		3	0	
Microvascular invasion	N	67	48	19	0.786	52	15	0.357
	P	16	12	4		14	2	
Intraneural invasion	N	73	53	20	0.864	62	11	0.003
	P	10	7	3		4	6	
HBV	N	73	51	22	0.146	57	16	0.347
	P	10	9	1		9	1	
Cirrhosis	N	67	48	19	0.786	53	14	0.847
	P	16	12	4		13	3	

[1]χ^2 test. NGF: Nerve growth factor; TrkA: Tropomyosin-receptor-kinase; HBV: Hepatitis B virus; N: Negative; P: Positive.

both lower expression, only NGF higher and only TrkA higher), subsequently investigated the correlation between these two groups. When expressions of NGF and TrkA were both higher, they had a significant relation with survival rate (P = 0.030), indicating that the NGF-TrkA pathway may be involved in the CCA progression in an autocrine loop way, as previously reported in breast cancer[14].

All the suspicious prognostic factors were enrolled in the Cox regression model to identify the independent prognostic factors, including tumor size, differentiation, T, N, M stage, satellites, macrovascular invasion, microvascualr invasion, intraneural invasion and NGF-TrkA double higher expression (Table 3). In IHCC, NGF and TrkA double higher expression was a significant prognostic factor (P = 0.010), meanwhile N stage was also defined as a prognostic parameter (P = 0.015). Other clinicopathologic parameters were not proved to be significantly related with prognosis in our experiments.

NGF-TrkA signaling pathway in CCA cell lines

It was previously indicated that overexpression of NGF-β may play an important role in progression of the perihilar CCA cell line QBC939[21], but TrkA role in the progression was not involved. To further explore the insight into the molecular mechanism and confirm the phenomenon found in our clinical study, we investigated NGF-TrkA signaling pathway in CCA cell lines with experiments *in vitro*. First of all, the levels of NGF and TrkA expressions were detected in three cell lines: HUCCT-1, RBE and HCCC9810 by immunoblotting (Figure 3A). Both NGF and TrkA can be detected in these three cell lines although the abundance was different. Interestingly, HUCCT-1 had the most NGF expression but the least TrkA expression. To activate TrkA receptor and detect downstream targeted molecules; we used 100 ng/mL recombinant NGF-β for stimulation. After starved in serum-free medium for 12 h and then stimulated for 5 or 15 min, phosphorylation levels of TrkA, AKT and ERK were detected by immunoblotting. Consistent with previous study, NGF can significantly activate the PI3K-AKT pathway and Ras-MAPK pathway in a time-related pattern (Figure 3B). The phosphorylated level of TrkA-pY490, AKT-pS473 and ERK-pT202/pY204 markedly increased with 5 min NGF stimulation, and 15 min stimulation had a more significant change, indicating that

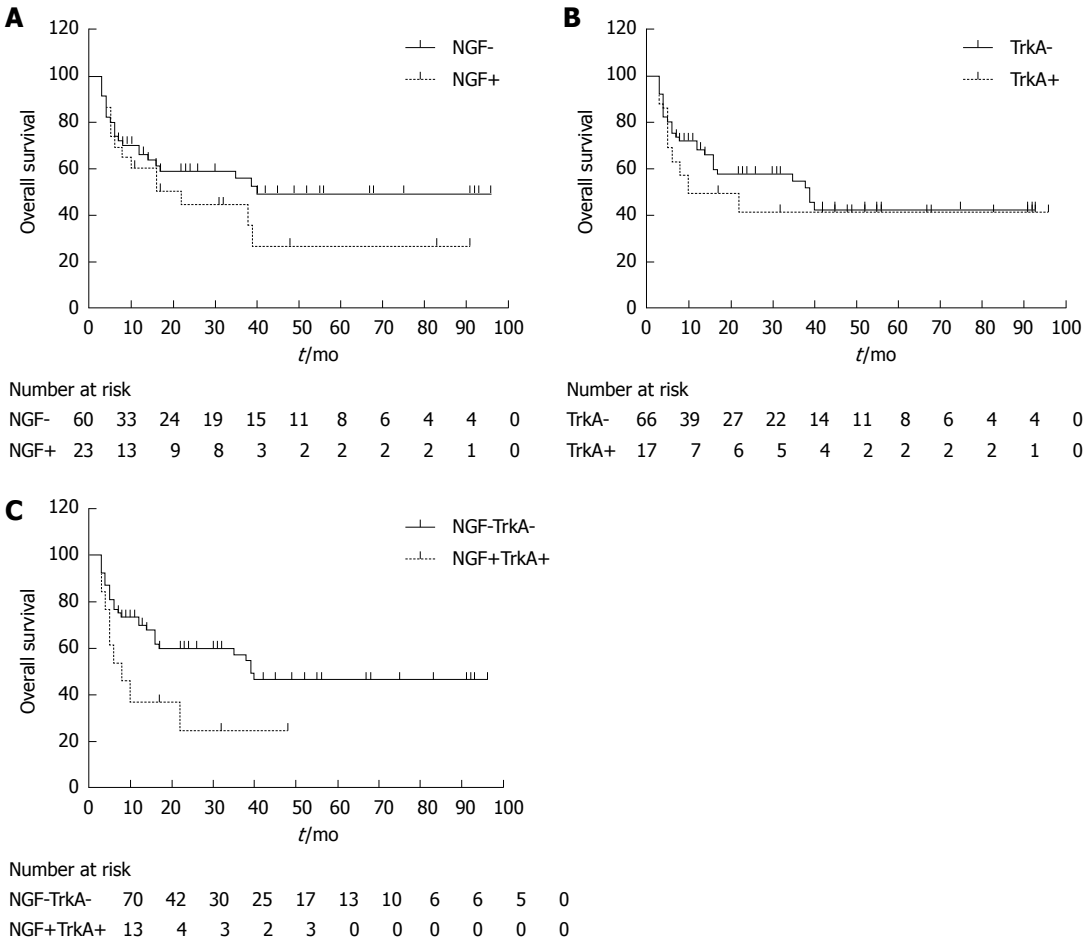

Figure 2 Correlations between overall survival rate and detected proteins. Expressions of NGF (A) and TrkA (B) have no significant association with prognosis of IHCC (P = 0.201 and 0.483, respectively). The group with NGF and TrkA double higher expression had poorer prognosis than the non-NGF/TrkA double higher group (C) (including both lower expression, only NGF higher and only TrkA higher, P = 0.003). NGF: Nerve growth factor; IHCC: Intrahepatic cholangiocarcinoma; TrkA: Tropomyosin-receptor-kinase.

NGF can trigger the PI3K-AKT and Ras-MAPK pathway by inducing TrkA phosphorylation. Furthermore, we knocked down TrkA by siRNA and evaluated the influence on cellular signaling and tumor progression. As expected, TrkA expression was knocked down successfully (Figure 3C), and the down-regulation of TrkA resulted in reduction of AKT-pS473 and ERK-pT202/pY204 phosphorylation, which suggested the crucial role of TrkA in NGF-induced MAPK and PI3K/AKT activation. Function assays including proliferation and invasion were carried out to identify NGF and TrkA role in CCA progression. RBE cells were starved in serum-free medium and then incubated with or without 100 ng/mL NGF-β for 48 h. The proliferation rate of control group without NGF stimulation was set as baseline and proliferation rate of other groups was evaluated by comparing with the ratio to baseline (Figure 3D). It was obvious that NGF could accelerate IHCC cells proliferation while TrkA knockdown significantly decreased this tendency, which demonstrating NGF/TrkA signaling pathway was required in IHCC proliferation.

Based on the founding that TrkA expression was associated with intraneural filtration, we speculated that TrkA was essential on invasion, so we performed transwell assay to evaluate the invasive ability of CCA cells. After split into matrigel-precoated well, RBE cells were then starved for 6 h and then activated by 100 ng/mL NGF-β, cell numbers were counted randomly in 8 visual fields and the number of control group without NGF-β was set as baseline. Similarly with proliferation, we can see that NGF-β markedly increased the invasive ability of RBE and TrkA knockdown reversed this increase significantly, which indicated that TrkA played crucial role in NGF-induced CCA invasion (Figure 3E).

DISCUSSION

The study on oncogenic role of NGF and its receptor was mostly focused in neural tumor such as neuroblastoma and sporadically reported in oral cancer, pancreatic cancer, colon cancer and breast cancer[25-28]. NGF was identified as a prognostic biomarker and mostly reported to be related with intraneural invasion, which is consistent with our study. Moreover, it is a breakthrough of CCA that NGF-β was identified to be associated with lymph and nerve invasion in perihilar cholangiocarcino-

Table 2 Univariate analysis of clinicopathologic features for overall survival

Clinicopathologic parameters		Median	Survival	*P* value[1]
Age	< 65 yr	46.2	39.5%	0.776
	⩾ 65 yr	59.9	60.3%	
gender	male	46.7	42.6%	0.364
	female	51.5	43.5%	
Tumor size	< 5 cm	57.4	50.2%	0.029
	⩾ 5 cm	40.6	36.1%	
Differentiation	Well	54.5	49.9%	0.587
	Moderately	47.8	40.4%	
	Poorly	43.6	40.7%	
T stage	T1	52.7	49.0%	0.411
	T2	58.9	60.0%	
	T3	33.0	23.4%	
	T4	0.0	0.0%	
N stage	N0	58.7	53.3%	0.002
	N1	17.6	14.0%	
M stage	M0	51.8	45.5%	0.004
	M1	7.8	0.0%	
TNM stage	Ⅰ	62.4	60.4%	0.004
	Ⅱ	73.0	77.8%	
	Ⅲ	46.6	36.8%	
	Ⅳ	17.2	13.6%	
Satellites	Negative	50.0	47.1%	0.132
	Positive	31.3	31.4%	
Macrovascular invasion	Negative	49.7	42.9%	0.290
	Positive	24.3	33.3%	
Microvascular invasion	Negative	49.5	43.9%	0.445
	Positive	44.7	41.8%	
Intraneural invasion	Negative	47.2	41.5%	0.678
	Positive	55.7	52.5%	
NGF	Negative	54.2	49.4%	0.201
	Positive	36.4	29.6%	
TrkA	Negative	48.9	42.7%	0.483
	Positive	44.6	41.6%	
NGF + TrkA	Negative	53.1	46.5%	0.030
	Positive	18.0	24.6%	

[1]Log-rank test. NGF: Nerve growth factor; TrkA: Tropomyosin-receptor-kinase.

Table 3 Multivariate analysis of clinicopathologic features

Factor	Category	HR	95%CI	*P* value[1]
Tumor size	< 5 cm	1.00		
	⩾ 5 cm	2.00	0.8-3.9	0.157
Differentiation	Well	1.00		
	Moderately	0.74	0.3-2.1	0.578
	Poorly	1.65	0.6-4.3	0.335
T stage	T1 + T2	1.00		0.550
	T3 + T4	1.25	0.6-2.6	
N stage	N0	1.00		0.015
	N1	3.15	1.3-7.9	
M stage	M0	1.00		0.200
	M1	2.18	0.7-7.2	
Satellites	Negative	1.00		0.533
	Positive	0.76	0.3-1.8	
Macrovascular invasion	Negative	1.00		0.245
	Positive	2.61	0.5-13.2	
Microvascualr invasion	Negative	1.00		0.092
	Positive	2.15	0.9-5.2	
Intraneural invasion	Negative	1.00		0.927
	Positive	0.95	0.3-2.9	
NGF + TrkA	Negative	1.00		0.010
	Positive	2.87	1.3-6.4	

[1]Cox proportional hazards regression. NGF: Nerve growth factor; TrkA: Tropomyosin-receptor-kinase.

ma, though the cases number was small and no prognostic data available[22]. However, more underlying molecular mechanism of why NGF related to poor prognosis of cancer need further investigation. NGF functioned mostly by interacting with its two receptors: p75NTR and Trk receptor. TrkA is distinguishing in the Trk receptors because it functions by autophosphorylating and activating of various signaling cascades. Proteins interacting directly with the TrkA include SHC, PLC 1, SH2B and IAPs, which can activate downstream signaling pathway, including RAS-MEKK-MAPK, and PI3K-PDK-AKT pathway. Activation of both MAPK and PI3K-AKT pathway can promote survival by affecting apoptosis-related protein BAD and BCL-2[29]. Moreover, NGF can promote intraneural invasion through activating STAT3 signaling[30]. Besides proliferation and invasion, NGF signaling pathway was proved to promote other oncogenic process like angiogenesis[31], which also need further investigation in CCA. In our study, we first found that NGF/TrkA signaling pathway can relate to poor prognosis in CCA and demonstrated that NGF/TrkA signaling pathway can promote CCA proliferation and invasion, which can provide new insight to CCA biomarker and progression, and help find a new molecular drug target for CCA.

Based on our clinical and experimental finding, we highly speculated that NGF and TrkA affected the progression of IHCC in an autocrine or paracrine way. The NGF which stimulated TrkA receptor of CCA cells could be secreted by neurocytes or by CCA cells themselves. Up-regulation of NGF in matrix could enhance invasive activity of CCA cells and promote the intraneural filtration, which can explain that TrkA expression was associated with intraneural invasion. NGF and TrkA network as paracrine loop could promote IHCC cells progression and finally lead to poor prognosis. In addition, more molecular insights into CCA tumorigenesis could be performed surrounding NGF and its receptors, and more experiments including animal model should be performed in the future.

The molecular significance of this signaling pathway was gradually revealed, which provides us a new inspiration for finding new chemical therapy. There are several potent small-molecular inhibitors of NGF and TrkA now, such as GW441756 or GNF-5837. More interestingly, the anti-NGF antibody tanezumab, which acts by sequestering NGF and preventing its binding to either of TrkA and p75, is in clinical trial for osteoarthritis and inflammation pain now. We hope our finding can increase the interest of NGF/TrkA signaling inhibitor as a potential chemical drug for CCA treatment.

In summary, for the first time, we demonstrated that NGF-TrkA double higher expression was associated with poor prognosis in IHCC and NGF-TrkA signaling pathway could promote IHCC cell line progression. This might provide a new insight into the molecular mechanism of IHCC progression. We hope our study could

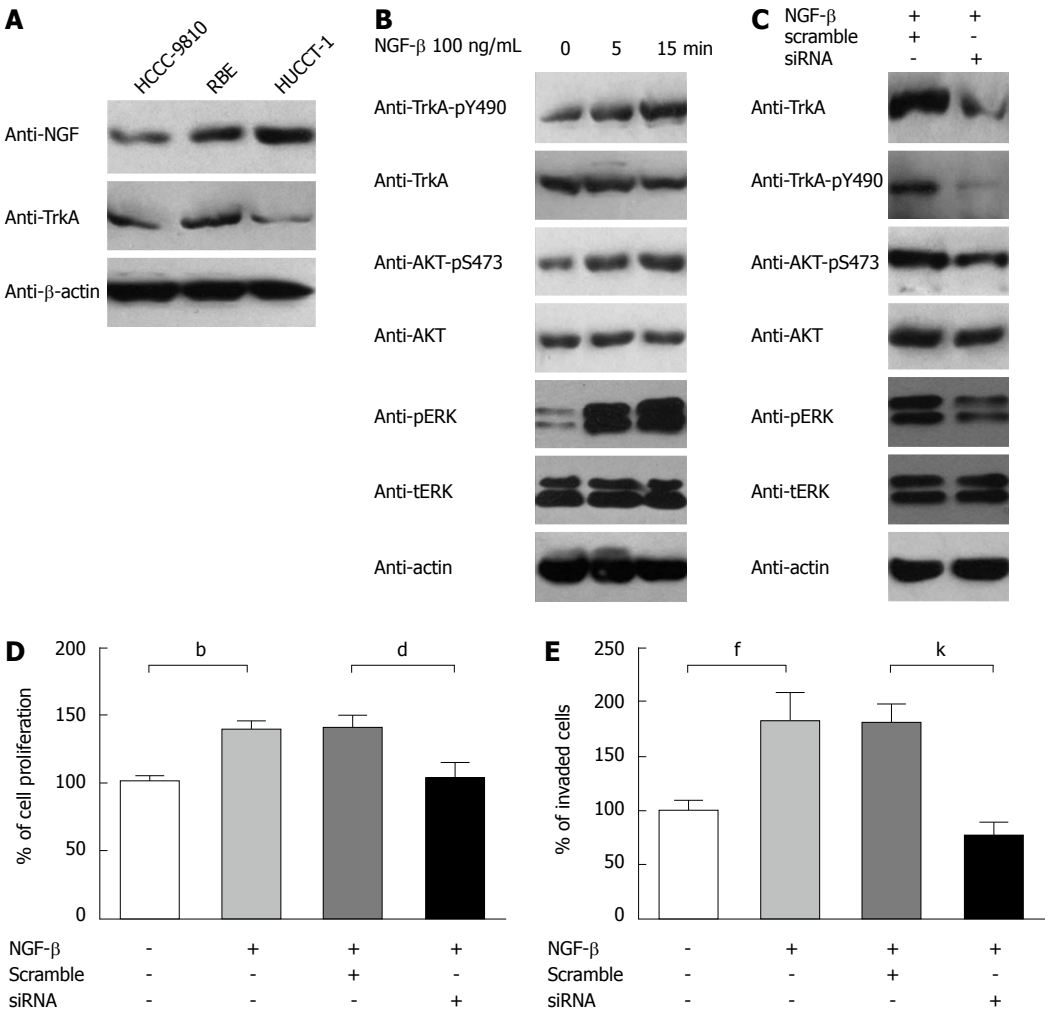

Figure 3 Nerve growth factor-tropomyosin-receptor-kinase signaling pathway can promote cholangiocarcinoma proliferation and invasion. A: NGF and TrkA expression in IHCC cell line HCCC9810, RBE and HUCCT-1; B: Phosphorylation level of TrkA, AKT and ERK notably elevated with NGF stimulation for different time (0, 5 or 15 min); C: After TrkA knocked down, phosphorylation level of TrkA, AKT and ERK decreases significantly; D: Proliferation of RBE was tested by MTT 48 h after siRNA transfection. NGF-β can promote RBE proliferation and TrkA knockdown reduces this tendency. Data were from three independent experiments and statistical analysis was performed by student *t* test, $^{b}P < 0.01$ between control and HCCC9810; $^{d}P < 0.01$ *vs* RBE and HUCCT-1; E: Invasive activity of RBE cells. RBE invasion is accelerated by NGF-β stimulation and reversed by TrkA knockdown. Cell numbers were counted under 200 × magnification. Data were from at least three independent experiments and statistical significance was measured by student *t* test, $^{f}P < 0.01$ between control and HCCC9810; $^{k}P < 0.01$ *vs* RBE and HUCCT-1. NGF: Nerve growth factor; IHCC: Intrahepatic cholangiocarcinoma; TrkA: Tropomyosin-receptor-kinase.

trigger the interest of NGF or TrkA as a potential drug target and help find new therapy of IHCC.

COMMENTS

Background

Nerve growth factor (NGF) and its receptor tropomyosin-receptor-kinase (TrkA) have been identified to be correlated to tumorgenesis and prognosis in several kinds of cancers. However,the importance of NGF-TrkA signaling pathway in intrahepatic cholangiocarcinoma (IHCC) is poorly elucidated.

Research frontiers

Recently, there is several high-level article focused on molecular classification of IHCC.

Innovations and breakthroughs

The authors found that NGF and TrkA double higher expression was an independent prognostic factor in IHCC; NGF/TrkA signaling pathway can activate MAPK and PI3K pathway in IHCC cell lines; NGF/TrkA signaling pathway played crucial role in IHCC proliferation and invasion.

Applications

Considering NGF/TrkA could promote IHCC progression, it may help develop new potential drugs for chemical therapy of IHCC.

Peer review

This article is original and while the nerve growth factor has been studied in relation to other types of cancers, cholangiocarcinoma data is sparse.

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Combined detection tumor markers for diagnosis and prognosis of gallbladder cancer

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Abstract

AIM: To clarify the value of combined use of markers for the diagnosis of gallbladder cancer and prediction of its prognosis.

METHODS: Serum cancer antigens (CA)199, CA242, carcinoembryonic antigen (CEA), and CA125 levels were measured in 78 patients with gallbladder cancer (GBC), 78 patients with benign gallbladder diseases, and 78 healthy controls using electrochemiluminescence. CA199, CA242, CEA, and CA125 levels and positive rates were analyzed and evaluated pre- and post-operatively. Receiver operator characteristic curves were used to determine diagnostic sensitivity and specificity of GBC. Survival time analysis, including survival curves, and multivariate survival analysis of a Cox proportional hazards model was performed to evaluate independent prognostic factors.

RESULTS: Serum CA242, CA125, and CA199 levels in the GBC group were significantly higher when compared with those in the benign gallbladder disease and healthy control groups ($P < 0.01$). With a single tumor marker for GBC diagnosis, the sensitivity of CA199 was the highest (71.7%), with the highest specificity being in CA242 (98.7%). Diagnostic accuracy was highest with a combination of CA199, CA242, and CA125 (69.2%). CA242 could be regarded as a tumor marker of GBC infiltration in the early stage. The sensitivity of CA199 and CA242 increased with progression of GBC and advanced lymph node metastasis ($P < 0.05$). The 78 GBC patients were followed up for 6-12 mo (mean: 8 mo), during which time serum CA199, CA125, and CA242 levels in the recurrence group were significantly higher than in patients without recurrence ($P < 0.01$). The post-operative serum CA199, CA125, and CA242 levels in the non-recurrence group were significantly lower than those in the GBC group ($P < 0.01$). Multivariate survival analysis using a Cox proportional hazards model showed that cancer of the gallbladder neck and CA199 expression level were independent prognostic factors.

CONCLUSION: CA242 is a marker of GBC infiltration in the early stage. CA199 and cancer of the gallbladder neck are therapeutic and prognostic markers.

Key words: Gallbladder cancer; Tumor marker; Combined detection; Diagnosis; Prognosis

Core tip: Detection of serum tumor markers is simple, and has become a common clinical method for tumor screening. However, when these markers are used individually for the diagnosis of gallbladder cancer, inconsistent results have been obtained. The results of the present study suggest that combined detection of serum cancer antigens cancer antigens (CA)125, CA199, and CA242 can increase the specificity of gallbladder cancer (GBC) diagnosis. CA242 could be regarded as a tumor marker of GBC infiltration in the early stage. Multivariate survival analysis showed that cancer of the gallbladder neck and CA199 expression levels were independent prognostic factors.

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INTRODUCTION

Gallbladder cancer (GBC) is one of the most common and aggressive malignant neoplasms of the biliary system. Early-stage GBC lacks typical clinical manifestations, leading to a poor 5-year survival[1-3]. Most patients are at the advanced stage at the time of diagnosis, and thus lose the chance of radical cure. It is therefore important to diagnose GBC earlier. Currently, the diagnosis of GBC mainly depends on non-invasive auxiliary imaging and invasive examination such as laparoscopy and biopsy. However, there is no ideal single tumor marker for the diagnosis and prognosis of GBC[4-6].

Tumor markers such as CEA, cancer antigens (CA) CA125, CA242, and CA199 have been widely used for the diagnosis of different types of cancer (*e.g.*, liver, gastric, colorectal, and pancreatic). However, when these markers are used individually for the diagnosis of GBC, inconsistent results have been obtained[7-10]. A recent study reported that S1P1 overexpression or ERp29 absence is related to carcinogenesis and progression, and are thus potential biomarkers for the early detection of gallbladder adenocarcinoma[11]. We therefore hypothesized as whether the combined use of tumor markers could avoid inconsistent results and increase the diagnostic sensitivity for GBC.

In this study, we detected serum levels of tumor markers CEA, CA125, CA242, and CA199 in 78 patients with GBC, 78 patients with benign gallbladder diseases, and 78 healthy controls. Our results showed that combined detection of these markers could increase the sensitivity for diagnosis and prognosis of GBC.

MATERIALS AND METHODS

Ethics

This study was performed in compliance with the Helsinki Declaration and according to the protocol approved by the Medical Ethics Committee of the authors' hospitals. All patients and participants were informed of the study and gave voluntary, signed informed consent.

Study subjects

A total of 234 subjects were enrolled in this study: 78 patients with GBC (30 male and 48 female), 78 patients with benign gallbladder diseases (cholecystitis, gallbladder polyps, and gallbladder stones) admitted to Yangpu District Central Hospital and Eastern Hepatobiliary Surgery Hospital (Shanghai, China), and 78 healthy individuals who underwent physical examinations in the same hospitals between January 2010 and September 2012. The 78 GBC patients ranged in age from 45 to 73 years, with an average of 58.7 years. Their body mass index ranged from 19 to 24 kg/m^2, with an average of 22. Cancer staging was performed according to the American Joint Committee on Cancer TNM staging system (7th Edition)/classification system for patients with gallbladder cancer (2010), which showed seven cases of phase Ⅱ GBC, 10 phase ⅢA, 33 phase ⅢB, 6 phase ⅣA, and 22 phase ⅣB. Of the 78 GBC cases, 73 were adenocarcinomas, including 8 highly differentiated adenocarcinomas, 11 poorly differentiated adenocarcinomas, and 54 moderately differentiated adenocarcinomas. The remaining five cases were carcinoid GBC in two, squamous cell carcinoma in two, and squamous Signet ring cell carcinoma in one. Of the 78 GBC cases, 37 occurred in the gallbladder neck, 23 in the body, 5 in the bottom, and 13 in the duct of gallbladder[12]. All GBC diagnoses were confirmed pathologically. None of the GBC patients had received radiotherapy, chemotherapy, or endocrine therapy before surgery. Metastasis occurred in 53 out of the 78 GBC cases, including 36 cases with adjacent lymph node (LN) metastasis, 17 with distal LN metastasis, and 25 without observable metastasis. The 78 patients in the benign gallbladder disease group included 35 men and 43 women, with a mean age of 55 ± 6.4 years (range: 48-70 years). The 78 healthy controls included 37 men and 41 women, with a mean age of 61.6 ± 6.7 years (range: 40-75 years). Serum CA199, CA242, CEA, and CA125 were detected before and after operation. Surgical modalities for the GBC group are shown in Table 1. Fasting cubital venous blood (5 mL) was drawn in the morning from each of the three groups and centrifuged at 4000 r/min. The supernatant was collected and preserved at -80 ℃ before use.

Detection of serum tumor markers

Serum CA199, CA242, CEA, and CA125 levels were detected by electrochemiluminescence immunoassay

Table 1 Surgical modalities and their relationship with tumor stage in the gallbladder cancer group

Surgical modality	TNM stage				
	Ⅱ	ⅢA	ⅢB	ⅣA	ⅣB
C + N	6	8	2		
C + WR + N	1		6		4
C + S4aS5 + N		1	7		3
C + ELH + N					1
C + ERH + N				1	
C + BD + N					
C + WR + BD + N		1	6		2
C + S4As5 + BD + N			4		2
C + CH + BD + N			3		1
C + S4aS5 + other + N				1	1
C + S4aS5 + other + N			5		
C + ERH + BD + other + N				1	1
HPD + N				1	
Palliative resection				1	2
Simple drainage				1	3
Pure exploration					2

BD: Bile duct resection; C: Cholecystectomy; CH: Central hepatectomy; ELH: Extended left hepatectomy; ERH: Extended right hepatectomy; HPD: Hepato-pancreaticoduodenectomy; N: Lymphadenectomy; Other: Other organ tissue resection; S4aS5: Liver resection of segments Ⅳa and Ⅴ; WR: Wedge resection of the gallbladder fossae.

(Cobas; Roche Diagnostics, Germany) at the Department of Biliary Surgery of the Eastern Hepatobiliary Surgery Hospital affiliated to the Second Military Medical University, Shanghai, China. The normal reference values were as follows: CEA ⩽ 10 μg/L, CA125 ⩽ 35 U/mL, CA242 ⩽ 15 U/mL, and CA199 ⩽ 39 U/mL.

Statistical analysis

The data were expressed as mean ± SD. Measurement data between groups were compared with the *t* test, while enumerative data were compared with the χ^2 test. The prediction value was calculated by receiver operating characteristic (ROC) curve analysis. Survival was analyzed by the Cox proportional hazards model. Statistical analysis was performed using SPSS version 17.0 statistical software (SPSS Inc., Chicago, IL, United States). All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Serum levels of CEA, CA125, CA199, and CA242

There were significant differences in the mean serum level and positive rate of CA125, CA199, and CA242 between the GBC and the other two groups ($P < 0.01$). There was no significant difference between the healthy control and benign gallbladder disease groups ($P > 0.05$). There was no significant difference in serum CEA, CA125, CA199, and CA242 levels with respect to age and sex in the GBC group ($P > 0.05$). The results are shown in Tables 2-4.

Table 2 Comparison of serum cancer antigen 199, cancer antigen 125, cancer antigen 242, and carcinoembryonic antigen levels

Group	*n*	MFI (CEA)	MFI (CA199)	MFI (CA125)	MFI (CA242)
Control group	78	3.93 ± 2.04	14.97 ± 8.91[b]	10.48 ± 6.38[b]	9.48 ± 3.43[b]
Benign disease	78	3.83 ± 1.85	15.17 ± 7.82[c]	12.99 ± 6.99[c]	10.19 ± 3.08[c]
GBC	78	9.36 ± 3.58	238.17 ± 346.36[b,c]	55.34 ± 81.78[b,c]	39.92 ± 45.9[b,c]

Results are mean ± SD. [b]$P < 0.01$ *vs* control group; [c]$P < 0.05$ *vs* benign disease group. MFI: Mean fluorescence intensity; CA: Cancer antigen; CEA: Carcinoembryonic antigen; GBC: Gallbladder cancer.

Table 3 Positive rates of carcinoembryonic antigen, cancer antigen 125, cancer antigen 242, and cancer antigen 199 *n* (%)

	GBC (*n* = 78)	Benign gallbladder disease (*n* = 78)	Healthy controls (*n* = 78)
CEA Pc	9 (11.5)	1 (1.2)	2 (2.5)
CA242 Pc	50 (64.1)[b]	2 (2.5)	1 (1.2)
CA125 Pc	35 (44.8)[b]	2 (2.5)	2 (2.5)
CA199 Pc	56 (71.7)[b]	4 (5.0)	3 (3.8)
Combination Pc	7 (8.9)	0 (0.0)	0 (0.0)

[b]$P < 0.01$ *vs* benign gallbladder disease and healthy control group. Combination = Carcinoembryonic antigen (CEA) + cancer antigen CA242 + CA125 + CA199. Pc: Positive cases; GBC: Gallbladder cancer.

Serum tumor markers in GBC patients with different clinicopathological features

According to the stage, location, and histological differentiation, the GBC group was subclassified by tumor location, stage, size, and pathological type for calculation of the positive rate of the four serum tumor markers using combined qualitative detection by a pathologist. The results showed that CA125, CA199, and CA242 levels in patients with gallbladder neck cancer were significantly higher than in those with cancer in the bottom portion. In addition, there were significant differences in serum CA125, CA199, and CA242 levels between GBC cases at different stages, tumor size, and differentiation (Table 4). Analysis of the sensitivity of the four serum tumor markers at different stages of GBC showed that the sensitivity of CA125, CA199, and CA242 strengthened gradually with the progression of clinical stages. There were significant differences between stages ⅣA, ⅣB, and Ⅱ ($P < 0.05$). The sensitivity of CA242 in stage Ⅱ GBC was significantly better than that of CA125 and CA199 ($P < 0.05$) (Table 5), and therefore, CA242 could be regarded as a tumor marker of GBC infiltration in the early stage.

GBC diagnostic value of a single vs three tumor markers

CA199 alone had the highest sensitivity of 71.7%, and CA242 alone had the highest specificity of 98.7% for the diagnosis of GBC. CA199 and CA242 had the most ex-

Table 4 Correlations between gallbladder cancer markers, tumor size, location, and staging

Group	*n*	MFI (CEA)	MFI (CA199)	MFI (CA125)	MFI (CA242)
Age (yr)					
≥ 50	45	9.5 ± 4.3	239.1 ± 324.6	55.9 ± 86.6	39.9 ± 42.9
< 50	33	9.2 ± 2.7	236.9 ± 319.4	54.6 ± 78.9	39.9 ± 47.3
Sex					
Male	30	9.3 ± 6.6	234.6 ± 356.9	54.8 ± 81.7	38.5 ± 47.1
Female	48	9.4 ± 3.8	240.4 ± 298.7	55.7 ± 89.5	40.8 ± 49.9
Tumor position					
Neck	37	8.5 ± 3.9	262.2 ± 177.8[b]	62.1 ± 47.0[b]	42.5 ± 45.9[b]
Body	23	5.2 ± 4.2	28.3 ± 20.7	56.9 ± 99.7	19.0 ± 21.6
Bottom	5	3.3 ± 2.6	27.6 ± 2.4[b]	14.5 ± 6.5[b]	8.5 ± 2.9[b]
Cystic duct	13	21.5 ± 25.1	622 ± 196.9	49.0 ± 32.9	81.7 ± 56.7
Staging					
Ⅱ	7	3.2 ± 2.2	66.1 ± 6.3[a]	17.7 ± 7.7[a]	8.4 ± 3.3[a]
ⅢA	10	2.0 ± 0.9	89.5 ± 8.2	47.3 ± 38.2	10.2 ± 9.9
ⅢB	33	6.9 ± 6.4	205.5 ± 33.9[a]	59.6 ± 114.1[a]	22 ± 22.6[a]
ⅣA	6	3.5 ± 3.3	383.6 ± 55.5[a]	58.1 ± 25.5[a]	55.2 ± 25.3[a]
ⅣB	22	19.9 ± 9.1	418.7 ± 316.5[a]	64.1 ± 50.5[a]	86.2 ± 56.9[a]
Tumor size					
> 5 cm	20	9.6 ± 2.2	768.1 ± 272.9[a]	82.9 ± 21.9[a]	81.8 ± 53.1[a]
≤ 5 cm	58	9.3 ± 1.2	55.4 ± 68.7[a]	45.8 ± 10.1[a]	25.5 ± 32.9[a]
Pathological type					
Highly differentiated	8	3.0 ± 2.0	68.9 ± 5.9[a]	16.7 ± 7.7[a]	8.7 ± 3.1[a]
Moderately differentiated	54	6.9 ± 5.9	142.3 ± 200.4	49.9 ± 9.9	33.8 ± 41.3
Poorly differentiated	11	3.5 ± 2.5	649.1 ± 209.1[a]	84.0 ± 29.4[a]	73.4 ± 58.9[a]
Carcinoid	2	20.9 ± 14.6	766.3 ± 330.5[a]	121.3 ± 45.2[a]	62.7 ± 9.4[a]
Squamous cell carcinoma	2	107.5 ± 69.1	334.4 ± 14.9[a]	116.0 ± 103.1[a]	108.5 ± 58.7[a]
Signet ring cell carcinoma	1	31	1000[a]	89.3[a]	69.3[a]

Results are mean ± SD. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group. MFI: Mean fluorescence intensity; CA: Cancer antigen; CEA: Carcinoembryonic antigen.

Table 5 Analyses of the sensitivity of tumor markers in different stages of gallbladder cancer *n* (%)

Clinical stages	Cases (*n*)	CEA	CA199	CA242	CA125
Ⅱ	7	1 (14.2)	3 (42.8)[a]	4 (57.1)[a,c]	2 (28.5)[a]
ⅢA	10	1 (10)	5 (50)	6 (60)	3 (30)
ⅢB	33	2 (6)	18 (54.5)	20 (60.6)	14 (42.4)
ⅣA	6	2 (33.3)	4 (66.6)	4 (66.6)	3 (50)
ⅣB	22	3 (13.6)	16 (72.7)	16 (72.7)	13 (59)

[a]$P < 0.05$ *vs* sensitivity of stage IVA, B; [c]$P < 0.05$ *vs* sensitivity of CA125 and CA199. CA: Cancer antigen; CEA: Carcinoembryonic antigen.

Table 6 Evaluation of diagnostic value of a single tumor marker in 78 gallbladder cancer cases

Diagnostic value	*n*	Sensitivity	Specificity	Positive likelihood ratio	Negative likelihood ratio
CEA	9	11.5%	97.4%	4.42	0.91
CA199	56	71.7%	96.1%	18.4	0.29
CA242	50	64.1%	98.7%	49.5	0.36
CA125	35	44.8%	96.2%	11.79	0.57

Sensitivity = true positive/patients × 100%; Specificity = true negative/normal × 100%; Positive likelihood ratio = sensitivity/(1 - specificity); Negative likelihood ratio = (1 - sensitivity)/specificity. CA: Cancer antigen; CEA: Carcinoembryonic antigen.

act validity (Table 6). ROC curves are shown in Figure 1. The sensitivity, specificity, and positive predictive values were: 91.0%, 94.9%, and 17.8%, respectively, when any of the three markers exceeded the critical value; 85.9%, 96.2%, and 22.6%, respectively, when any two of the three markers exceeded the critical values; and 69.2%, 100%, and 100%, respectively, when all three markers exceeded the critical values. The sensitivity was 8.9% when all four markers exceeded the critical values. These results suggested that diagnosis of GBC based on combined detection of the tumor markers could increase the specificity, but not sensitivity, of diagnoses (Table 7).

Table 7 Analyses of different combinations of markers in gallbladder cancer diagnosis

Group	*n*	1 item (+)	2 item (+)	3 item (+)	4 item (+)
Normal	78	4 (5.1)	3 (3.8)	0 (0)	0 (0)
Gallbladder cancer (GBC)	78	71 (91.0)	67 (85.9)	54 (69.2)	7 (8.9)
Positive likelihood rate		17.8%	22.6%	100%	100%

Correlations of CEA, CA125, CA199, and CA242 expression with LN metastasis in GBC

Serum CEA, CA125, CA199, and CA242 expression in GBC patients with and without LN metastasis was compared. Serum CA125, CA199, and CA242 levels in patients with LN metastasis were significantly higher than those in patients without LN metastasis ($P < 0.01$). Serum CA125, CA199, and CA242 levels in patients with distal LN metastasis were significantly higher than those in pa-

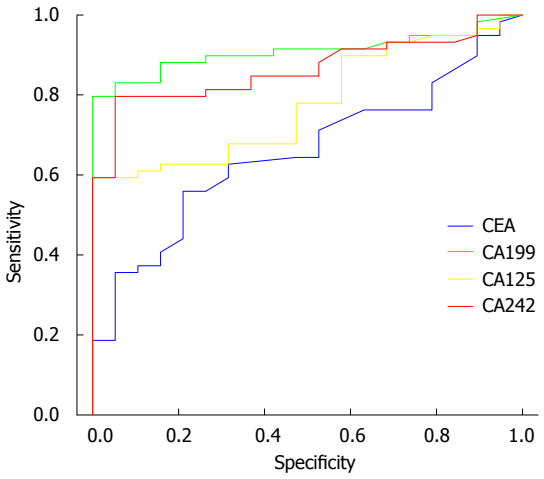

Figure 1 Receiver operating characteristic curve. Receiver operating characteristic curves showing diagnostic performance of cancer antigens (CA) CA199, CA125, CA242, and carcinoembryonic antigen (CEA). The sensitivity of CA199 was the highest (71.7%) and the specificity of CA242 was the highest (98.7%).

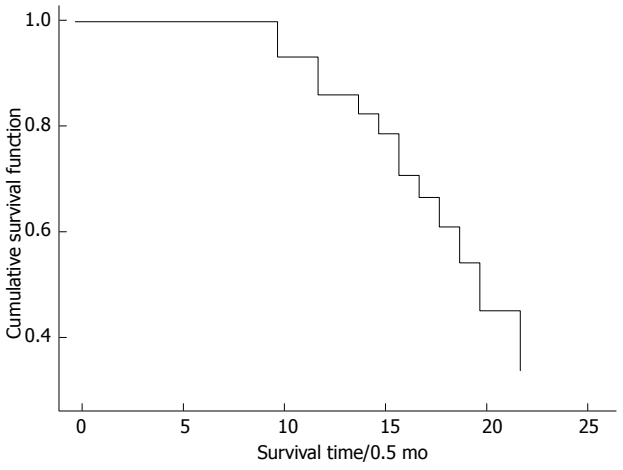

Figure 2 Analysis of overall survival curves. Overall survival curves for 78 patients with gallbladder cancer (GBC). Kaplan-Meier survival curves in GBC patients with a Cox proportional hazards model for multivariate regression analysis.

Table 8 Correlations between carcinoembryonic antigen, cancer antigen 125, cancer antigen 242, and cancer antigen 199 expression and lymph node metastasis

Indicators	No LN metastasis (n = 25)	Adjacent LN metastasis (n = 36)	Distal LN metastasis (n = 17)
CEA, μg/L	7.5 ± 3.4	9.5 ± 5.9	9.8 ± 3.6
CA199, U/mL	122.2 ± 117.2	237.4 ± 189.5[b]	491.2 ± 222.5[a,b]
CA242, U/mL	9.5 ± 2.9	38.5 ± 15.7[b]	58.7 ± 29.6[a,b]
CA125 U/mL	13.5 ± 16.5	43.6 ± 37.9[b]	61.8 ± 67.8[a,b]

Results are mean ± SD. [a]P < 0.05, [b]P < 0.01 *vs* patients without lymph node (LN) metastasis. CA: Cancer antigen; CEA: Carcinoembryonic antigen.

Table 9 Carcinoembryonic antigen, cancer antigen 125, cancer antigen 242, and cancer antigen 199 expressions during follow-up

Groups	n	CEA	CA199	CA242	CA125
Non-recurrence group	22	7.34 ± 2.14	78.65 ± 86.43[d]	12.41 ± 1.25[d]	11.96 ± 2.37[d]
Recurrence group	8	9.34 ± 3.04	219.74 ± 321.63[b]	34.54 ± 8.38[b]	53.88 ± 8.2[b]

Results are mean ± SD. [b]P < 0.01 *vs* non-recurrence group; [d]P < 0.01 *vs* gallbladder cancer (GBC) group. CA: Cancer antigen; CEA: Carcinoembryonic antigen.

tients with adjacent LN metastasis (P < 0.05) (Table 8).

Follow-up results

During the follow-up period from 6 mo to 1 year, 17 patients died, and contact was lost with 31 for various reasons. The survival time in each patient was defined as the interval between the date of definitive resection and the date of last follow-up or death. In the 30 patients who completed the follow-up study, six experienced recurrence. Serum CA125, CA199, and CA242 levels in the recurrence group were significantly higher than those in the non-recurrence group 6 mo post-operatively (P < 0.01). Serum CA125, CA199, and CA242 levels in the non-recurrence group were significantly lower than those in the GBC group pre-operatively (P < 0.01) (Table 9). Multivariate survival analysis using the Cox proportional hazards model showed that cancer of the gallbladder neck and CA199 expression level were independent prognostic factors (Table 10). Survival curves are shown in Figure 2.

Table 10 Results of Cox proportional hazards model for multivariate regression analysis

Prognostic factor	Parameter estimate	Wald χ^2	P value	HR	95%CI
CEA	-0.043	0.498	0.48	0.957	0.849-1.080
CA199	0.005	12.076	0.001	1.005	1.002-1.009
CA242	-0.007	0.386	0.535	0.993	0.972-1.015
CA125	0.003	0.714	0.398	1.003	0.996-1.009
Surgical modality	0.569	2.450	0.118	1.766	0.866-3.599
LN metastasis	1.191	3.276	0.07	3.291	0.906-11.957
Position (neck)	-1.578	6.358	0.012	0.206	0.061-0.704
Age	-0.010	0.073	0.787	0.990	0.922-1.063
Sex	0.796	2.508	0.113	2.216	0.828-5.932

Multivariate survival analysis using the Cox proportional hazards model for independent prognostic factors of gallbladder cancer (GBC). P < 0.05 was considered statistically significant. CA: Cancer antigen; CEA: Carcinoembryonic antigen; LN: Lymph node.

DISCUSSION

The incidence of GBC has increased in recent years worldwide. How to treat GBC, assess therapeutic effect, evaluate prognosis, and predict post-operative recurrence in the early stage has aroused increasing attention in both clinical studies and practice[13,14]. In this study, we analyzed

the diagnostic value of the four common clinical serum tumor markers, CA242, CEA, CA125, and CA199 for GBC, and compared serum levels of these markers in patients with GBC, patients with benign gallbladder disease, and healthy controls. Serum levels of CA242, CA125, and CA199 were significantly higher in the GBC than the benign gallbladder disease and healthy control groups. Serum levels of the four markers were not significantly different between the benign disease and healthy control groups (P = 0.592-0.953). Although we also analyzed the effect of age and sex on serum levels of the four markers in GBC diagnosis, these factors did not change the GBC diagnostic value of the markers. In addition, we investigated correlations of the four markers with the clinicopathological features of GBC. CA242, CA125, and CA199 levels in cancer of the gallbladder neck were significantly higher than in cancer located in the bottom portion. In addition, there were significant differences in the serum level of CA242, CA125, and CA199 between GBC with different differentiation. CA242, CA125, and CA199 increased with the progression of the GBC stage. There was a significant difference in the sensitivity of the four markers between stages ⅣA, ⅣB, and Ⅱ. The results showed that the sensitivity of CA242, CA125, and CA199 (but not CEA) increased gradually with the progression of the clinical stages. The sensitivity of CA199 was the highest, reaching 71.7%, with the highest specificity being that of CA242 at 98.7%. When CA242, CA125, and CA199 exceeded the critical values, the sensitivity was 69.2%, the specificity was 100%, and the positive predictive rate was also 100%. When all four markers exceeded the critical values, the sensitivity was 8.9%. Diagnostic accuracy was highest with a combination of CA242, CA125, and CA199. These findings suggest that the combined use of these markers for the diagnosis of GBC could increase the specificity of diagnosis, but not the sensitivity. The optimal cut-off values of the markers determined by ROC curve analysis could improve the diagnosis of GBC when used together with these markers, resulting in their optimal application and promoting the clinical screening and diagnosis of GBC[15].

We found that serum CA242, CA125, and CA199 levels were significantly higher in patients with LN metastasis than in those without. Serum CA242, CA125, and CA199 levels in patients with distal LN metastasis were significantly higher than in those with adjacent LN metastasis. Most researchers believe that CA199 is a better marker of malignant tumors. Serum CA199 is elevated most obviously in tumors of the digestive system, pancreas, and biliary tract[16]. CA199 is not only a diagnostic indicator, but also a predictor of the therapeutic effect and prognosis of GBC. However, CA199 is not specific for GBC. Therefore, CA199 should be combined with other imaging tests to diagnose GBC[17]. CA125 is a good marker for the diagnosis of cholangiocarcinoma. Qu *et al*[18], Wu *et al*[19] and Shukla *et al*[20] showed that CA125 has a relatively high specificity because it is rarely affected by serological levels of inflammation and liver stones. Our research was not exactly consistent with that of Shukla *et al*[20]. Combined use of CEA and CA199 or CA125 can improve the diagnosis of cholangiocarcinoma[15,21-26], which is consistent with our study. The positive rate of serum CA242 was high in GBC patients but not in patients with benign gallbladder diseases or the normal control group. Tao *et al*[27] have suggested that combined detection of α-fetoprotein (AFP) and CA242 could improve the sensitivity and specificity of the diagnosis of cholangiocarcinoma. Rana *et al*[28] reported that CA242 was better than CEA and CA199 as a tumor marker for the diagnosis of GBC. The expression of CEA is high in most gastrointestinal tumors[29,30]. Stefanović *et al*[31] found that CEA expression was significantly increased in GBC. However, Vij *et al*[32] suggested that CEA and AFP had little value for the diagnosis and prognosis of GBC. Our study also showed that CEA had limited value for the diagnosis and prognosis of GBC. In terms of a single marker for the diagnosis of GBC, CA199 has the highest sensitivity with relatively low specificity, but cannot be used alone as an effective tumor marker to identify GBC. CA242 has the highest specificity, probably because it is rarely affected by the serological level of liver inflammation and stone disease, nor is it affected by the low expression in the pancreatic duct system and pancreatic juice siltation. However, the expression of CA242 is high in almost all malignant tumors, and therefore cannot be used to differentiate between GBC and pancreas cancer[33].

The follow-up study in 30 cases showed that serum CA242, CA125, and CA199 levels in the recurrence group were significantly higher than those in the non-recurrence group. Pre-operation, these levels in the non-recurrence group were lower than those in GBC group as a whole. Multivariate survival analysis using the Cox proportional hazards model showed that cancer of the gallbladder neck and CA199 expression level were independent prognostic factors[34-36].

Post-operative tumor recurrence and metastasis are major causes of death in GBC patients. To achieve a comprehensive and accurate understanding about the probability of post-operative recurrence and metastasis of GBC, efforts have been made to explore more effective clinical predictors. The tumor-related indicators in all individuals cannot be detected systemically and comprehensively due to limited economics and techniques[37], and therefore joint detection of specific tumor markers is of great significance[38].

In summary, the expression of CA242, CA125, and CA199 has an important application in assessing LN metastasis, monitoring recurrence, and clinical staging of tumors. Joint detection of CA242, CA125, and CA199 may prove to be useful for the diagnosis of GBC, assessing therapeutic effects, and predicting a prognosis. CA242 could be a marker of GBC infiltration in the early stage. Cancer of the gallbladder neck and CA199 expression level were independent prognostic factors.

COMMENTS

Background

Early diagnosis and resection of gallbladder cancer (GBC) offers a chance of cure. It is therefore important to diagnose GBC early. However, there is no ideal single tumor marker for the diagnosis and prognosis of GBC. Moreover, when these markers are used individually for the diagnosis of GBC, inconsistent results have been obtained. The aims of this study were to determine whether the combined use of tumor marker cancer antigens (CA)199, CA242, carcinoembryonic antigen (CEA), and CA125 could increase sensitivity for the diagnosis of GBC, as well as to determine the clinicopathological prognostic factors affecting survival and recurrence.

Research frontiers

The authors analyzed the published literature on combined tumor marker detection for early diagnosis, treatment, and prognosis of GBC, and found no other related or similar studies.

Innovations and breakthroughs

Combined detection of serum CA242, CA125, and CA199 had the highest specificity for the diagnosis of GBC. Diagnostic accuracy was highest with a combination of CA242, CA125, and CA199. CA242 can be regarded as a tumor marker of GBC infiltration in the early stage. The sensitivity of CA199 and CA242 increased with the progression of GBC stage, advanced lymph node metastasis, and recurrence. A Cox proportional hazards model for multivariate survival analysis showed that cancer of the gallbladder neck and CA199 expression level were independent prognostic factors.

Applications

The results suggest that combined detection of tumor markers can increase the specificity of GBC diagnosis. CA242 can be regarded as a tumor marker of GBC infiltration in the early stage. CA199 or cancer of the gallbladder neck is a valuable marker for assessing therapeutic effect and predicting prognosis.

Peer review

This was a well-designed study and a well-written paper on the role of tumor markers in the diagnosis of GBC. GBC has a dismal prognosis, and radical surgical resection offers the only chance of cure, but is possible in only a small proportion of patients due to advanced disease, and the rate of recurrence is high. This study contributes to the early diagnosis of the disease, influencing its prognosis.

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META-ANALYSIS

Effect of precut sphincterotomy on post-endoscopic retrograde cholangiopancreatography pancreatitis: A systematic review and meta-analysis

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Abstract

AIM: To conduct a systemic review and meta-analysis to investigate the role of early precut technique. Multiple randomized controlled trails (RCTs) have reported conflicting results of the early precut sphincterotomy.

METHODS: MEDLINE/PubMed, EMBASE, Cochrane Central Register of Controlled Trials and Database of Systematic Reviews, and recent abstracts from major conference proceedings were searched (June 2013). Randomized and non-randomized studies comparing early precut technique with prolonged standard methods were included. Pooled estimates of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP), cannulation and adverse events were analyzed by using odds ratio (OR). Random and fixed effects models were used as appropriate. Publication bias was assessed by funnel plots. Heterogeneity among studies was assessed by calculating I^2 measure of inconsistency.

RESULTS: Seven randomized and seven non-randomized trials met inclusion criteria. Meta-analysis of RCTs showed a decrease trend for PEP with early precut sphincterotomy but was not statistically significant (OR = 0.58; 95%CI: 0.32-1.05; P = 0.07). No heterogeneity was noted among the studies with I^2 of 0%.

CONCLUSION: Early precut technique for common bile duct cannulation decreases the trend of post-ERCP pancreatitis.

Key words: Early precut; Endoscopic retrograde cholangiopancreatography cannulation; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Randomized controlled trials; Meta-analysis

Core tip: Multiple trials are available in literature, but still the optimal timing of precut sphincterotomy is debatable. We conducted systemic review and meta-analysis to explore the effect of early precut sphincterotomy on post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis with emphasis on analysis of optimal timing of precut sphincterotomy. Our meta-analysis showed that early precut sphincterotomy decreases the odds of post-ERCP pancreatitis, particularly if done within 5-10 min of failed cannulation without compromising cannulation rates or increasing other complications.

Choudhary A, Winn J, Siddique S, Arif M, Arif Z, Hammoud GM, Puli SR, Ibdah JA, Bechtold ML. Effect of precut sphincterotomy on post-endoscopic retrograde cholangiopancreatography pancreatitis: A systematic review and meta-analysis. *World J Gastroenterol* 2014; 20(14): 4093-4101 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4093.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4093

INTRODUCTION

Common bile duct (CBD) cannulation is a prerequisite for any biliary therapeutic intervention in endoscopic retrograde cholangiopancreatography (ERCP). Even in experienced hands, cannulation may be difficult in 10%-20% of cases based on patient and procedure related factors[1]. Precut sphincterotomy is one of the rescue techniques in these difficult cannulations. The term "precut" refers to action of performing sphincterotomy before CBD access is achieved. In this technique, a cut is made at orifice and extended cephalad for a variable distance to expose CBD opening, commonly referred to as papillotomy. However, another technique has been described in which a cut is made few millimeters above papillary orifice and extended downwards, commonly referred to as fistulotomy. Both these techniques help in facilitating CBD access in difficult cannulation.

Although precut is a rescue step in difficult cannulation, it is considered a risk factor for ERCP adverse events, particularly pancreatitis. Several large prospective studies[2-6] and a meta-analysis[7] have identified precut sphincterotomy as an independent risk factor for post-ERCP pancreatitis (PEP), irrespective of the number of attempts. Therefore, the current practice is that precut sphincterotomy is used as a salvage measure when multiple attempts of cannulation have failed *via* standard methods. However, recent larger studies have identified repeated cannulation attempts with standard approach as a risk factor for PEP, rather than precut sphincterotomy itself[8,9]. Several randomized[10-16] and non-randomized studies[17-23] of small sample sizes have evaluated the role of early precut sphincterotomy *vs* repeated attempts at cannulation with or without guidewire for the prevention of PEP with varying results. Because of the varied results and small sample sizes of the studies, we performed a systematic review and meta-analysis to compare adverse events and cannulation rates in early precut *vs* standard conventional methods in CBD cannulation.

MATERIALS AND METHODS

Study selection

Articles and abstracts comparing early institution of precut sphincterotomy *vs* standard methods of cannulation with sphincterotome with or without guidewire for CBD cannulation and PEP were selected. Studies comparing two different modalities for CBD cannulation with using precut sphincterotomy as a rescue mechanism and/or case series were excluded. The literature search was restricted to adult patients. There were no language restrictions. Both full length and abstract publications were selected.

Literature search and identification of primary studies

All articles comparing early precut sphincterotomy with standard approach for CBD cannulation were searched irrespective of language, publication status (articles or abstracts), or results. A three-stage search strategy was adopted and implemented. First, a search of MEDLINE, EMBASE, and Cochrane Central Register of Controlled Trials using PubMed and Ovid as search engines (1966-June 2013). The search terms used were precut for CBD cannulation, precut sphincterotomy, precut papillotomy, precut endoscopic retrograde cholangiopancreatography, post-ERCP pancreatitis, post-ERCP pancreatitis and prevention, and/or post-ERCP pancreatitis risk factors. Second, reference lists of retrieved articles, reviews, and meta-analyses were scanned for additional articles. Third, a manual search of abstracts submitted to the Digestive Disease Week, American College of Gastroenterology, and United European Gastroenterology Week (2005-2013) was performed.

Data extraction

Data extraction was independently performed by two investigators (Choudhary A and Winn J) and reviewed by a third investigator (Bechtold ML) for agreement. The two independent investigators extracted data from each study using a common data extraction form. Details of study design (randomization/blinding/concealment), number of subjects and dropouts, methods of precut, timing of precut in precut group, total duration and attempts in conventional group, use of guidewire and stents, inclusion and exclusion criteria, other adverse events, cannulation rate, and PEP were evaluated. All studies were assigned a quality score based upon the Jadad scale, with five being of high quality and zero being of poor quality[24]. Disagreements were discussed and resolved by consensus.

Statistical analysis

Statistical pooling of the data was done for randomized controlled trials (RCTs). Data from non-randomized studies were excluded from the statistical pooling. Primary outcome was the incidence of PEP. Secondary outcomes were the cannulation rates and overall adverse events. All data was analyzed according to both per-protocol and intention-to-treat analysis. The effects of early precut application were analyzed by calculating pooled estimates of PEP, cannulation rates, and other adverse events. Separate analyses were performed for each outcome by using odds ratio (OR) or weighted mean difference (WMD). Random or fixed effects models were used as appropriate. A statistically significant result was indicated by 95%CI not including 1 and a *P*-value of < 0.05. Whenever statistical significance was

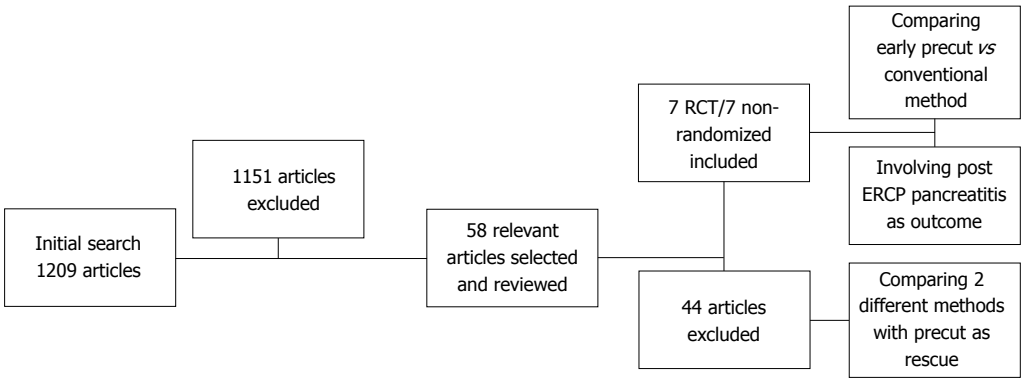

Figure 1 Article identification and selection algorithm. RCT: Randomized controlled trial; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 1 Quality analysis of included randomized trials

Study	Year	Center	Country	Type of RCT	Allocation concealment	Intention to-treat	Jadad score
Cennamo *et al*[13]	2009	Single	Italy	Non blinded	A	Yes	3
Tang *et al*[15]	2005	Single	Canada	Non blinded	A	Yes	3
Khatibian *et al*[14]	2008	Single	Iran	Non blinded	B	Yes	3
de Weerth *et al*[12]	2006	Single	Germany	Non blinded	B	Yes	3
Zhou *et al*[11]	2006	Single	China	Non blinded	B	N/A	2
Manes *et al*[10]	2009	Multi	Italy	Non blinded	A	Yes	3
Swan *et al*[16]	2013	Single	Australia	Non blinded	A	N/A	2

Allocation concealment: A: Adequate; B: Unclear; N/A: Data not available; RCT: Randomized controlled trail.

detected, an absolute risk reduction with 95%CI and the number needed-to-treat (NNT) with 95%CI were calculated. Rev Man 5.2 software was utilized for statistical analysis of the data. Subgroup and cumulative analysis were performed to the evaluate the effect of timing of precut sphincterotomy, study size, study quality, different methods of precut, fellows' participation and role of pancreatic duct stent in precut application, and PEP. Publication bias was assessed by funnel plot. Heterogeneity among studies was assessed by calculating I^2 measure of inconsistency with $P < 0.05$ being considered statistically significant.

Non-randomized studies: Data from the non-randomized studies were also extracted as described above. Retrospective or prospective non-randomized studies were included. Case series were excluded from the analysis. Primary and secondary outcomes analyzed were similar as stated above.

RESULTS

Initial search resulted in 1209 relevant articles. Out of these, 58 were selected for final review as shown in Figure 1. All articles were independently reviewed by two authors (AC and JW). Seven randomized controlled (n = 1032) (Figure 1) and seven non-randomized studies (n = 3548) met the inclusion criteria and were selected for final review and analysis. Both randomized and non-randomized studies were analyzed separately. Table 1 shows the details and Jadad scores for the selected RCTs (5 = excellent quality, 0 = poor quality). All studies were of adequate quality (Jadad scores ≥ 2). Included studies were conducted in different parts of the world, including three studies in Europe, two in Asia, and one each in Canada and Australia respectively. All studies except one were single-center studies. Table 2 represents baseline characteristics of subjects in the included randomized studies[10-16]. Mean age of subject ranged from 55.9-71 years. The majority of studies had a predominant female subject population. Indications for ERCP varied in all trials with CBD stone as predominant indication in majority of trials ranging from 10.3% to 74.5%. Four trials included subjects with sphincter of Oddi dysfunction (SOD)[10,14-16] with one trial having 21.2% of subjects with a possible SOD diagnosis[14]. Five trials included subjects with malignancy leading to obstructive jaundice[10-13,15]. All studies excluded patient who had previous sphincterotomy, history of acute pancreatitis at the time of procedure, and patients with altered anatomy. Table 3 showed interventions done in various trials. Timing for precut sphincterotomy varied among the studies. Two studies[12,14] used precut as initial method of cannulation in early precut group, whereas two trials[13,16] used five minutes of failed cannulation as a marker for difficult cannulation before precut sphincterotomy. The other three trials used 10 min or multiple attempts of failed cannulation[10,11,15] before precut sphincterotomy. In standard cannulation group, additional time of 10-20 min are allowed in different trials before proceeding to precut as one of the rescue methods or aborting the procedure. Methods of precut sphincterotomy varied among differ-

Table 2 Baseline patient characteristics of included trials

Study	Patients (*n*)		Age (year in mean)		Female		Malignant jaundice		CBD stone		SOD	
	E	Std	E	Std	E	Std	E	Std	E	Std	E	Std
Cennamo *et al*[13]	36	110	68[1]	71[1]	55.5%	53.6%	33.3%	25.4%	66.6%	74.5%	0	0
Tang *et al*[15]	32	30	64.6	67.2	53.1%	53.3%	34.3%	33.3%	21.9%	13.3%	3.1%	6.7%
Khatibian *et al*[14]	106	112	56.6	55.9	53.8%	67%	N/A	N/A	51.9%	68.8%	21.2%	12.8%
de Weerth *et al*[12]	145	146	66	64	66%	66%	40%	23%	46.8%	59.5%	N/A	N/A
Zhou *et al*[11]	43	48	62.7	64.3	39.5%	39.5%	30.2%	25%	20.9%	22.9%	N/A	N/A
Manes *et al*[10]	77	74	66	65	35%	35.1%	33.7%	40.5%	53.2%	41.9%	0	2.7%
Swan *et al*[16]	39	34	59	57	72%	66%	N/A	N/A	10.3%	17.6%	5.1%	8.8%

[1]Age in median year. E: Early pre cut; Std: Standard method of cannulation; N/A: Data not available; CBD: Common bile duct; SOD: Sphincter of oddi dysfunction.

Table 3 Interventions in various trials and pancreatitis definitions

Study	Precut timing	Timing for std cannulation	Type of pre cut	Use of GW	PD stent/PP	PEP criteria	Fellow involvement
Cennamo *et al*[13]	5 min of failed cannulation or 3 pancreatic duct cannulation	20 min post randomization	Papillotomy	Yes	No	Consensus Criteria	No
Tang *et al*[15]	12 min of failed cannulation	15 min post randomization	Papillotomy	No	No	Consensus Criteria	Yes
Khatibian *et al*[14]	Immediate precut	15 min post randomization	Fistulotomy	Yes	No	Consensus Criteria	No
de Weerth *et al*[12]	Immediate precut	20 min post randomization or 3 pancreatic cannulation	Papillotomy	Yes	N/A	Consensus Criteria	No
Zhou *et al*[11]	10 min of failed cannulation or 3 pancreatic duct cannulation	N/A	Papillotomy and fistulotomy	Yes	N/A	N/A	N/A
Manes *et al*[10]	10 min of failed cannulation or 5 pancreatic duct cannulation	10 min post randomization	Fistulotomy	Yes	No	Consensus Criteria	N/A
Swan *et al*[16]	5 min of failed cannulation or 2 pancreatic duct cannulation	10 min post randomization	Papillotomy	Yes	Yes[1]	Consensus Criteria	Yes

[1]Subjects with inadvertent PD cannulation. GW: Guidewire; PP: Pharmacological prophylaxis for post ERCP pancreatitis; N/A: Data not available; PD: Pancreatic duct; PEP: Post ERCP pancreatitis; ERCP: Endoscopic retrograde cholangiopancreatography.

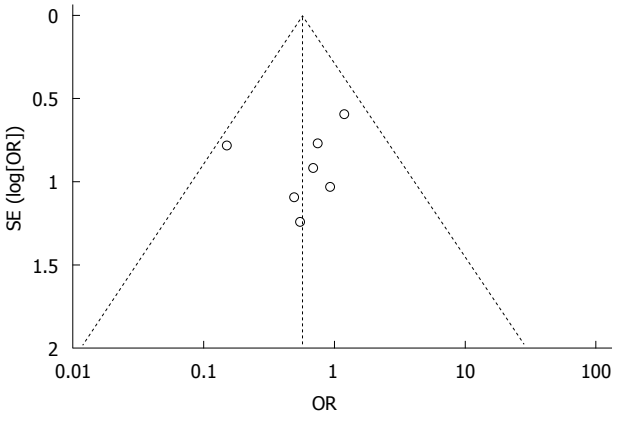

Figure 2 Funnel plot for publication bias.

ent studies. Four studies[10,13,15,16] used papillotomy, two studies used fistulotomy[10,14], and one study used both fistulotomy and papillotomy[11]. All trials except one[15] used guidewire for cannulation. Only one trial used pancreatic duct (PD) stent for PEP[16] prevention in subjects with inadvertent PD cannulation. Additionally, only one trial[13] provided separate data about adverse events when precut was applied as one of the rescue methods in conventional group. In two trials[15,16] fellows participated in the procedure (Table 4).

Publication bias was evaluated by funnel plot with no significant publication bias identified (Figure 2).

Post-ERCP pancreatitis

All trials, except one trial[11] used the consensus definition[25] for defining PEP. In this trial, no information was provided about definition of PEP. All seven trials provided data regarding PEP. PEP was documented in 19 of 478 (3.9%) patients with early precut group *vs* 34 of 554 (6.1%) patients in the standard cannulation group. On pooled analysis, a trend toward decreased PEP was noticed with early precut sphincterotomy but did not reach statistical significance (OR = 0.58; 95%CI: 0.32-1.05, P = 0.07, Figure 3). No heterogeneity was noted among the studies with I^2 of 0%.

Subgroup and cumulative analyses were performed to evaluate the effect of various factors on PEP. Subgroup analysis was performed based on timing and methods of precut application, quality of study, use of PD stent, and fellows' participation during the procedure. Cumulative analysis was performed based on the timing of precut application, year of studies, size, and quality of studies.

Table 4 Outcome of various studies

Study	Primary cannulation rates		ITT		Overall complication		Pancreatitis		Cholangitis		Bleeding		Perforation		Need for second ERCP	
	E	Std	E	Std	E	Std	E	Std	E	Std	E	Std	E	Std	E	Std
Cennamo *et al*[13]	33/36	104/110	36/36	110/110	3/36	7/110	1/36	6/110	N/A	N/A	1/36	1/110	1/36	0/110	3/36	6/110
Tang *et al*[15]	24/32	22/30	31/32	28/30	4/32	3/30	2/32	2/30	1/32	0/30	1/32	0/30	0/32	0/30	0/32	1/30
Khatibian *et al*[14]	88/106	100/112	105/106	111/112	3/106	3/112	2/106	3/112	0/106	0/112	0/106	0/112	1/106	0/112	18/106	12/112
de Weerth *et al*[12]	145/145	145/146	145/145	146/146	3/145	5/146	3/145	4/146	N/A	N/A	0/145	1/146	0/145	0/146	0/145	1/146
Zhou *et al*[11]	39/43	36/48	N/A	N/A	4/43	7/48	1/43	2/48	0/43	2/48	1/43	0/48	0/43	0/48	N/A	N/A
Manes *et al*[10]	63/77	66/74	71/77	71/74	11/77	16/74	2/77	11/74	N/A	N/A	5/77	2/74	0/74	1/77	14/77	8/74
Swan *et al*[16]	34/39	29/34	N/A	N/A	9/39	8/34	8/39	6/34	N/A	N/A	1/39	2/34	0/39	0/34	N/A	N/A

E: Early pre cut; Std: Standard method of cannulation; N/A: Data not available; ITT: Intention to treat; ERCP: Endoscopic retrograde cholangiopancreatography.

Timing of precut

On sub-group analysis, no significant effect was noticed with immediate precut sphincterotomy[12,14] (OR = 0.73, 95%CI: 0.23-2.33, $P = 0.59$) but a trend toward decreased PEP was noticed with pooled analysis of trials with precut sphincterotomy after 5-12 min[10,11,13,15,16] of failed cannulation (OR = 0.53, 95%CI: 0.26-1.07, $P = 0.08$). Similarly, a trend for PEP reduction was noticed on analysis of trials with precut sphincterotomy within 5-10 min (OR = 0.49, 95%CI: 0.23-1.04, $P = 0.07$)[10,11,13,16].

On cumulative analysis based on timing of precut sphincterotomy, no significant effect was noticed with immediate[12,14] (OR = 0.73, 95%CI: 0.23-2.33, $P = 0.59$) or immediate and within 5 min of failed cannulation[12-14,16] (OR = 0.85, 95%CI: 0.40-1.80, $P = 0.67$); however, a definite trend toward decreased PEP was noted with immediate and within 10 min of failed cannulation[10-14,16] (OR = 0.55, 95%CI: 0.29-1.03, $P = 0.06$, Table 5).

Methods of precut

Four studies[12,13,15,16] used papillotomy, whereas two studies[10,14] used fistulotomy. One trial[11] used both fistulotomy and papillotomy, hence was excluded from this subgroup analysis. On pooled analysis, fistulotomy significantly decreases the odds of PEP (OR = 0.27, 95%CI: 0.09-0.82, $P = 0.02$). The absolute risk difference was 5% (95%CI: 1%-10%) and the NNT was 20. On the other hand, papillotomy failed to show significant odds reduction for PEP (OR = 0.89, 95%CI: 0.41-1.92, $P = 0.77$, Table 6).

Use of stent for PEP prophylaxis

One study[16] used pancreatic duct stent as PEP prophylaxis, but only in cases where pancreatic duct was inadvertently cannulated. On pooled analysis, after excluding this study, a significant reduction in odds of pancreatitis in early precut group in comparison to standard cannulation group was noted (OR = 0.45, 95%CI: 0.22-0.91, $P = 0.03$).

Fellow participation

In two trials[15,16], fellows participated in the study and initiated the procedure. Sub-group analysis on exclusion of these two trials showed significant odds reduction of PEP with early precut sphincterotomy (OR = 0.40, 95%CI: 0.19-0.88, $P = 0.02$) with no heterogeneity.

Quality of study

Subgroup analysis of studies with Jadad score of 3 or more[10,12-15] showed significant less odds of pancreatitis with early precut sphincterotomy in comparison to standard cannulation group (OR = 0.44, 95%CI: 0.21-0.93, $P = 0.03$) with no heterogeneity.

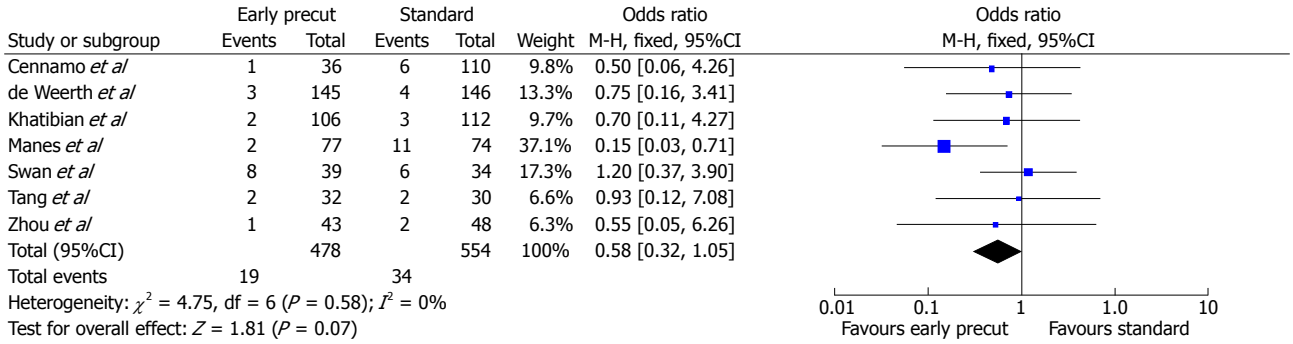

Figure 3 Forrest plot demonstrating post endoscopic retrograde cholangiopancreatography pancreatitis.

Table 5 Subgroup and cumulative analysis for timing of precut sphincterotomy

Timing of precut	OR	*P* value
Subgroup analysis		
Immediate	0.73; 95%CI: 0.23-2.33	0.59
5-10 min	0.49; 95%CI: 0.23-1.04	0.07
5-12 min	0.53; 95%CI: 0.26-1.07	0.08
Cumulative analysis		
Immediate	0.73; 95%CI: 0.23-2.33	0.59
Immediate and within 5 min	0.85; 95%CI: 0.40-1.80	0.67
Immediate and within 10 min	0.55; 95%CI: 0.29-1.03	0.06

Table 6 Odd's ratio for outcomes

Outcomes	OR	*P* value
Methods of precut		
Fistulotomy	0.27; 95%CI: 0.09-0.82	0.02
Papillotomy	0.89; 95%CI: 0.41-1.92	0.77
Study quality		
High quality study	0.44; 95%CI: 0.21-0.93	0.03
Sample size > 100	0.39; 95%CI: 0.17-0.89	0.02

Year and size of study

No difference in results were noted on cumulative analysis based on year of study, but a significant odds reduction for PEP was observed on analysis of studies with Jadad score of 3 or more with study size more than 100 subjects[10,12-14] (OR = 0.39; 95%CI: 0.17-0.89, *P* = 0.02). No heterogeneity was noted.

Cannulation rate

All seven trials provided data regarding primary cannulation rates. Pooled per-protocol and intention-to-treat analysis of cannulation rates showed no difference between both groups (OR = 0.90; 95%CI: 0.59-1.37, *P* = 0.62, Figure 4). No significant heterogeneity was noted.

Need for second ERCP

All except two trials[11,16] provided data on need for second ERCP. Pooled analysis was performed and showed no difference for the need for second ERCP for cannulation (OR = 1.54; 95%CI: 0.91-2.61, *P* = 0.11). No heterogeneity was noted.

Overall adverse events

The adverse events provided in all trials were PEP, perforation, cholangitis, and bleeding. On pooled analysis, trend towards lower overall adverse events was noted in early precut group (7.7%) in comparison to standard cannulation group (8.8%) but this did not reach statistical significance (OR = 0.80; 95%CI: 0.50-1.27, *P* = 0.34). Only three perforations were noted in all trials involving 1032 patients, two of which occurred in precut group. On pooled analysis, no significant difference was noticed between both groups (OR = 1.96; 95%CI: 0.40-9.56, *P* = 0.40) with no significant heterogeneity. Similar results were noted for bleeding and cholangitis (OR = 1.54; 95%CI: 0.59-3.99, *P* = 0.38; OR = 0.68; 95%CI: 0.11-4.25, *P* = 0.68) respectively, with no significant heterogeneity.

Systemic review of non-randomized studies

We also analyzed the data from non-randomized studies (Table 7). Seven studies met the inclusion criteria (*n* = 3548). Three studies were published as manuscripts and four as abstracts. All studies were done recently. Sample size varied from 57 to 2004 patients. In two studies[21,22], precut was applied immediately, in three other studies[17,18,23], precut was applied after 10 failed attempts at biliary cannulation, and in one study[20], it was applied after 10 min of failed cannulation or five inadvertent pancreatic duct cannulations. A statistically significant reduction of PEP with early precut application was noted in two studies[20,23]. In the other five studies, a low incidence of PEP was noted with early precut but did not reach statistical significance. The range of PEP varied from 0%-10.3% in early precut group *vs* 5.8%-42.8% in standard group.

DISCUSSION

Successful bile duct cannulation remains the cornerstone for therapeutic ERCP. Multiple innovative techniques and tools have been developed for safe and successful cannulation. However, despite these techniques, cannulation may be unsuccessful in 10%-20% of cases based on experience of endoscopist and multiple procedural and patient related factors[1]. In these difficult cannulations, PEP is considered one of the most feared adverse

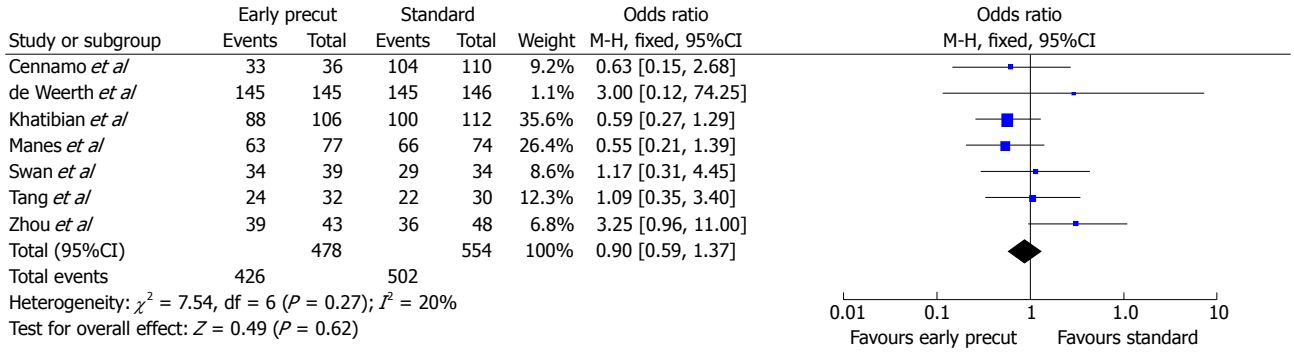

Figure 4 Forrest plot demonstrating cannulation rates.

Table 7 Summary of non-randomized studies

Study (lead author/year)	Country	Publication type	Mean age	Females	Pre cut application	*n*	Pancreatitis		*P*
							EP	Std	methods
Giussani *et al*[17], 2008	Italy	Abstract	N/A	N/A	< 10 cannulation attempts	804	8.3%	11.6%	NS
Testoni *et al*[18], 2010	Italy	Article	N/A	50.20%	< 10 cannulation attempts	308	7.6%	15.4%	NS
Xinopoulos *et al*[19], 2009	N/A	Abstract	N/A	N/A	N/A	134	8.3%	8.5%	NS
Madacsy *et al*[20], 2009	Hungary	Article	57	91%	10 min of failed cannulation or 5 pancreatic duct cannulation	57	0%	42.8%	< 0.05
Ayoubi *et al*[21], 2009	Italy	Abstract	68.6 (median)	58.9%	Immediate precut	173	1.1%	5.8%	NS
De La Mora-Levy *et al*[22], 2011	Mexico	Abstract	N/A	N/A	Immediate precut	68	10.3%	10.2%	NS
Testoni *et al*[23], 2011	Italy	Article	N/A	49.7%	< 10 cannulation attempts	2004	3.3%	14.3%	< 0.05

N/A: Data not available; NS: Non significant; EP: Early pre cut; Std: Standard method of cannulation.

events with varied incidence. Precut sphincterotomy is considered as a last resort and is one of the rescue techniques, given its implication as an independent risk factor for PEP irrespective of number of attempts[7]. Contrary to this, a few recent trials have suggested that increasing number of attempts at biliary cannulation is a risk factor for PEP rather than precut itself[8,9]. In one of the trials, risk of PEP has already increased to 14% as a result of multiple failed cannulation attempts at the time of precut sphincterotomy[26]. Recently, Bailey *et al*[27] demonstrated incremental increase in the incidence of PEP with increasing cannulation attempts; 11.5% with 10 to 14 attempts and 15% with > 15 attempts.

In our meta-analysis, a trend toward decreased PEP and other adverse events with similar cannulation rates with early precut sphincterotomy was observed. Although the overall pooled analysis did not reach statistical significance, on subgroup analysis of high quality studies, a significant odds reduction for PEP was observed with early precut sphincterotomy in comparison to standard approach. Similar results were observed on subgroup and cumulative analyses based on size of study as well as study quality. These results should be interpreted with caution, as studies used different time periods before considering precut sphincterotomy, with timing varying from immediate precut[12,14] to after 12 min of failed cannulation[15]. Though, above result of decrease trend in PEP is similar to previous meta-analysis[28] but in this current meta-analysis, an attempt was made by conducting cumulative and subgroup analysis to find the optimal timing before considering the cannulation as difficult and attempting precut sphincterotomy. In subgroup analysis, no difference was observed between immediate precut sphincterotomy and standard method of cannulation, but a trend toward decreased PEP was observed in subgroup analysis if precut sphincterotomy was performed within 5-10 min[10,11,13,16] of failed cannulation ($P < 0.06$). Similar results were observed on cumulative analysis. No difference was observed on immediate precut sphincterotomy ($P = 0.59$) and 5 min of failed cannulation ($P = 0.67$), but trend toward decreased PEP was observed with 10 min of failed cannulation ($P = 0.06$), similar to the subgroup analysis (Table 5). Interestingly, in contrast to popular belief, immediate precut application did not increase the risk of PEP on subgroup and cumulative analyses. PEP was noted in 1.9% in immediate pre-cut group in comparison to 2.7% in the standard group ($P = 0.59$).

Although in the present meta-analysis, a significant odds reduction of PEP with early precut sphincterotomy was noted on subgroup analysis of trials without fellow participation and fistulotomy, these results are based on exclusion of trials with smaller number of subjects (for fellow participation)[15,16] and analysis of only two trials (for fistulotomy)[10,14]. Overall incidences of other adverse events reported were very low in all the trials for both the groups and no difference was noted on pooled analysis. Similarly, cannulation rates were similar in both

groups but not all the trials presented the data about the time required for cannulation.

In our analysis, we did not include data from non-randomized studies, however, similar results were observed on review of non-randomized studies with decreasing trend in PEP with early precut sphincterotomy, except in two trials in which a significant reduction of PEP was observed with early precut sphincterotomy.

Strengths of the present meta-analysis include inclusion of both randomized and non-randomized trials to explore literature, no significant heterogeneity for any of the analyzed outcomes was noted, and inclusion of good quality trials conducted in different parts of the world. Additionally, for the first time, an attempt was made to explore optimal timing of precut sphincterotomy. There are several limitations of the present study which include the following. First, relatively few numbers of studies were available to adequately conduct subgroup analysis to determine optimal timing and techniques of precut sphincterotomy. Second, no recommendations can be made about the role of precut sphincterotomy in the high-risk population as trials have heterogeneous subject population undergoing ERCP with only a few trials including subjects with SOD. Finally, the role of PD stenting and other pharmacologic interventions for PEP prophylaxis, which are considered as routine and standard of care in difficult cannulation as shown in previous meta-analysis and randomized trial[29,30], is unclear with early precut sphincterotomy. Future multicenter randomized controlled trials are needed not only to determine the optimal timing and technique of precut sphincterotomy, but also to explore the role of prophylactic PD stenting and other pharmacological interventions in combinations with precut sphincterotomy in difficult cannulations.

In conclusion, early precut sphincterotomy decreases the trend of PEP, particularly if done within 5-10 min of failed cannulation without compromising cannulation rates or increasing other adverse events.

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COMMENTS

Background

Despite multiple innovative techniques and tools, common bile duct cannulation remains difficult in 10%-20% of cases. Precut sphincterotomy is considered as last resort, given its implication as an independent risk factor for post-endoscopic retrograde cholangiopancreatography pancreatitis (PEP) irrespective of number of attempts. Recently few published trials compared the outcomes and risks of early precut with varied results.

Research frontiers

Multiple randomized and non-randomized trials with small sample sizes have analyzed the effects of early precut sphincterotomy with varied results. In the field of PEP and precut sphincterotomy, the research hot spot is to find the optimal time of precut application in difficult cannulation cases.

Innovations and breakthroughs

Early precut sphincterotomy decreases the trend of PEP in comparison to conventional methods of cannulation, especially if done within 5-10 min of failed cannulation without compromising cannulation rates or increasing other adverse effects.

Applications

Precut sphincterotomy can be safely performed within 5-10 min of failed conventional cannulation methods.

Terminology

Early precut sphincterotomy decreases the trend of PEP without increasing any other adverse effects or compromising cannulation rates.

Peer review

This is a well conducted meta-analysis on the outcomes and risk of PEP with early precut sphincterotomy.

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CASE REPORT

Simultaneous liver mucinous cystic and intraductal papillary mucinous neoplasms of the bile duct: A case report

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Abstract

Cystic hepatic neoplasms are rare tumors, and are classified into two separate entities: mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms of the bile duct (IPMN-B). We report the case of a 56-year-old woman who presented with abdominal pain and jaundice due to the presence of a large hepatic multilocular cystic tumor associated with an intraductal tumor. Partial hepatectomy with resection of extrahepatic bile ducts demonstrated an intrahepatic MCN and an intraductal IPMN-B. This is the first report of the simultaneous occurrence of these two histologically distinct entities in the liver.

Key words: Liver; Mucinous cystic neoplasm; Intraductal papillary mucinous neoplasm of the bile duct

Core tip: Cystic hepatic neoplasms are rare tumors, and are classified as mucinous cystic neoplasms (MCN) characterized by intracystic septae, ovarian-like stroma, and no communication with bile ducts; and intraductal papillary mucinous neoplasms of the bile duct (IPMN-B) characterized by intraductal growth, dilated bile ducts, and papillary projections. We report a 56-year-old woman diagnosed with a large multilocular cystic tumor in the liver and in dilated extrahepatic bile ducts that were two histologically distinct cystic tumors. This is the first report of the simultaneous occurrence of MCN and IPMN-B. We present diagnostic considerations, therapeutic approaches, and the prognosis of cystic tumors of the liver.

Budzynska A, Hartleb M, Nowakowska-Dulawa E, Krol R, Remiszewski P, Mazurkiewicz M. Simultaneous liver mucinous cystic and intraductal papillary mucinous neoplasms of the bile duct: A case report. *World J Gastroenterol* 2014; 20(14): 4102-4105 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4102.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4102

INTRODUCTION

Cystic hepatic neoplasms are categorized into two separate entities: hepatic mucinous cystic neoplasms (MCN) and intraductal papillary mucinous neoplasms of the bile duct (IPMN-B)[1]. MCNs are rare tumors that account for less than 5% of all cystic liver lesions. MCNs are mucin-containing septate cystic tumors, covered with cuboidal

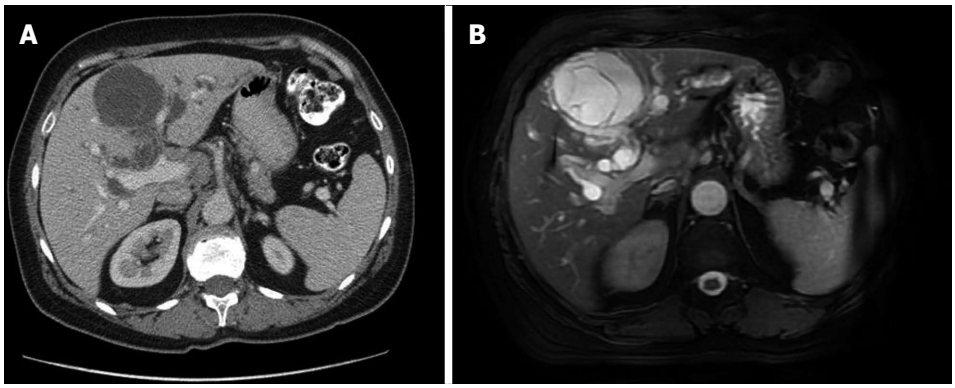

Figure 1 Computed tomography scan and T1-weighted magnetic resonance imaging. A: Computed tomography scan (portal phase) shows a hepatic cystic tumor with thin contrast-enhanced septum and dilated intrahepatic bile ducts; B: T1-weighted magnetic resonance imaging of the tumor presents a hyperintense, multiseptal cystic lesion that does not communicate with the bile ducts.

or columnar epithelium that present with ovarian-like stroma between the inner epithelial lining and the outer connective tissue capsule. This type of tumor has no communication with the biliary tree. IPMN-Bs are a newly described pathologic entity characterized by intraductal microscopic or macroscopic papillary growth, forming a cystic tumor that communicates with the bile ducts and shows no ovarian-like stroma. IPMN-Bs may produce a large amount of mucin, resulting in extensive dilatation of the bile ducts[2,3]. These two types of cystic neoplasms may present variable stages of dysplasia, at times progressing *via* carcinoma *in situ* to invasive cholangiocarcinoma. We present a unique case of the simultaneous occurrence of both types of hepatic cystic neoplasms.

CASE REPORT

A 56-year-old woman presented at the outpatient clinic complaining of upper abdominal discomfort. Upper digestive tract endoscopy revealed *Helicobacter pylori* (*H. pylori*)-positive duodenitis. The patient was given *H. pylori* eradication therapy, but showed no remarkable improvement. After two months she developed jaundice and mild itching. Ultrasonography showed a large multilocular cystic tumor located in the median part of the left hepatic lobe (segment IVB).

The liver function tests demonstrated significantly increased serum liver enzyme levels: γ-glutamyl transpeptidase, 776 IU/L; alkaline phosphatase, 281 IU/L; alanine transaminase, 198 IU/L; aspartate transaminase, 125 IU/L; and serum bilirubin, 6.4 mg/dL. Carcinoembryonic antigen 19-9 and alfa-fetoprotein were normal. Hydatid cystic disease was excluded by negative serologic tests.

Computed tomography and magnetic resonance imaging revealed an 8-cm cystic tumor occupying hepatic segments IVB (Figure 1). The tumor had a thin capsule and septations that showed enhancement with intravenous contrast medium. The intrahepatic bile ducts were dilated, especially within the left hepatic lobe. The hepatic duct and proximal part of the common bile duct were dilated to 25 mm. Magnetic resonance cholangiopancreatography demonstrated significant dilation of the peripheral biliary ducts in the left lobe of the liver and cystic-like dilation of the proximal part of the extrahepatic biliary duct with a sharp change in the lumen diameter at the midportion of the common bile duct (Figure 2A).

Based on suspected malignancy, surgical resection was selected. Preoperative endoscopic retrograde cholangiopancreatography revealed a large oval tumor within the hepatic bile duct, presenting as a bulky filling defect (Figure 2B). On intraoperative inspection, we observed a large intrahepatic cystic tumor in the median part of the left hepatic lobe and a palpable floating soft mass in the main biliary duct that almost completely obstructed biliary flow. After incising the confluence of the hepatic ducts, the cystic tumor entering the left hepatic duct was visualized. The bile was thick, suggesting mucinous content. The two tumors were resected by left hemihepatectomy, excluding segment I, with concomitant resection of the extrahepatic bile ducts, followed by anastomosis of the right hepatic duct with Roux-Y intestinal loop. The postoperative course was uneventful. Neither tumor had recurred after 7 mo of postoperative follow-up.

The tumors were grossly and microscopically separate. Microscopically, the intrahepatic tumor was a MCN composed of a single-layered mucin-producing epithelium that showed low-grade dysplasia without signs of malignancy (Figure 3A and B). The presence of ovarian-like stroma was confirmed by positive immunostaining for progesterone and estrogen receptors (Figure 3C). The tumor originating from the main hepatic duct was composed of epithelial papillary proliferations with focal ulcerations (Figure 4). None of the histologic features suggested a malignant change. The fibrous intratumoral interstitium lacked any mesenchymal or ovarian-like stroma.

DISCUSSION

The term IPMN-B refers to a recently classified subtype of biliary neoplasm, which is considered the biliary counterpart of pancreatic IPMN because they share several clinicopathologic features. It is a slow-growing tumor with relatively indolent clinical behavior and a lower malignant potential than ductal cell carcinoma and mucinous

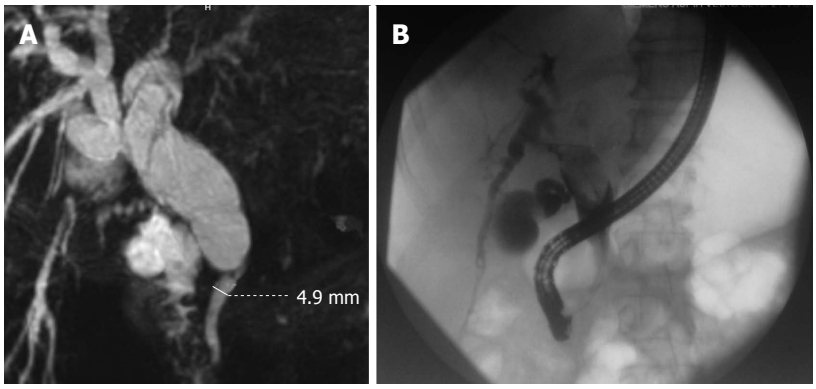

Figure 2 Magnetic resonance and preoperative endoscopic retrograde cholangiopancreatography. A: Magnetic resonance cholangiopancreatography shows dilated main hepatic and common bile duct with a sharp change in diameter from 25 to 5 mm; B: Preoperative endoscopic retrograde cholangiopancreatography shows marked dilation of the extrahepatic biliary ducts with a contrast-filling defect caused by an oval intraductal tumor.

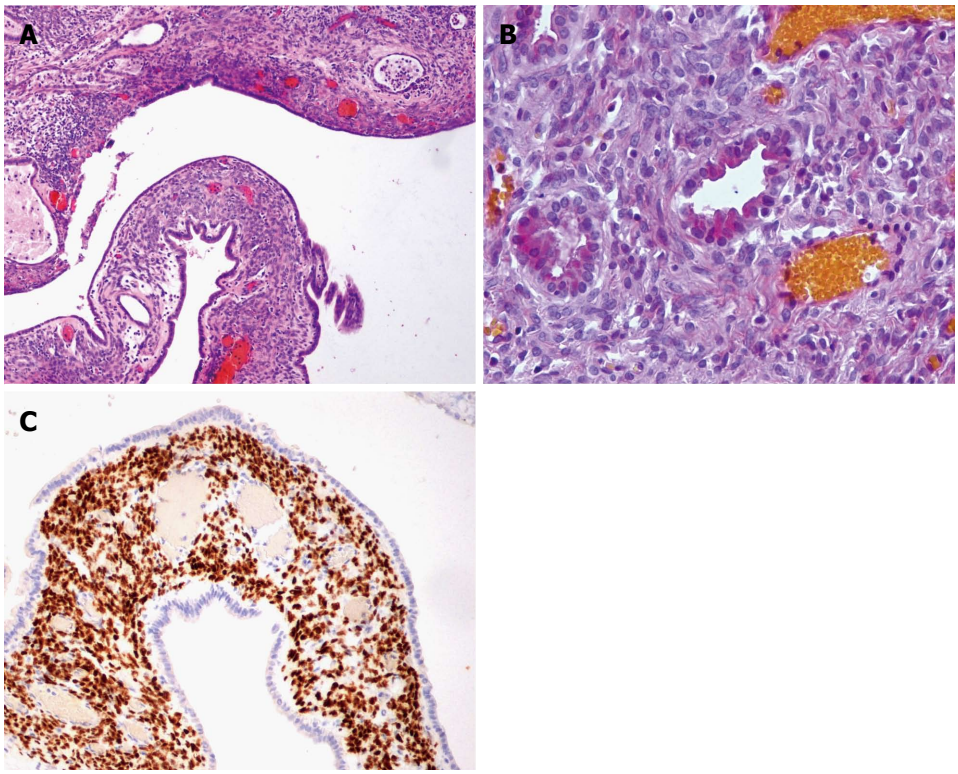

Figure 3 Histology of the intrahepatic tumor. A: The cyst is covered with a single layer of cuboidal epithelial cells showing low grade dysplasia (hematoxylin-eosin stain, × 100 magnification); B: The tumor produces mucin; C: Ovarian-like stroma staining positively for estrogen receptor.

cystadenocarcinoma[4,5]. IPMN-B has been reported with several different names, such as mucin-producing intrahepatic cholangiocarcinoma, biliary papillary tumor, mucin-producing bile duct tumor, and biliary intraductal papillary mucinous neoplasia. An excellent prognosis with a 5-year survival rate higher than 80% can be expected after complete resection of the tumor with negative margins[6].

IPMN-B is associated with other malignancies (5.6% of cases), including hepatocellular carcinoma, gallbladder cancer, cervical cancer, and salivary gland cancer[4]. Several reports on the coincidence of IMPN-B with its pancreatic counterpart have been published[7,8]. To the best of our knowledge, this is the first case in the English-language literature of the simultaneous occurrence of IPMN-B and MCN in the liver. In contrast to MCNs, which are more frequently detected in women, IPMN-Bs occur in both sexes and are usually located in the left hepatic duct or perihilar region presenting as a multicystic tumor with a grape-like appearance[3,9]. Ovarian-like stroma is considered essential for the diagnosis of MCN, whereas papillary-like projections and intraductal growth are necessary for the diagnosis of IPMN-B. Missed diagnosis of IPMN-B may be common. In our patient, preoperative endoscopic retrograde cholangiopancreatography was more useful in the diagnosis of the intraductal tumor than routine computed tomography and magnetic

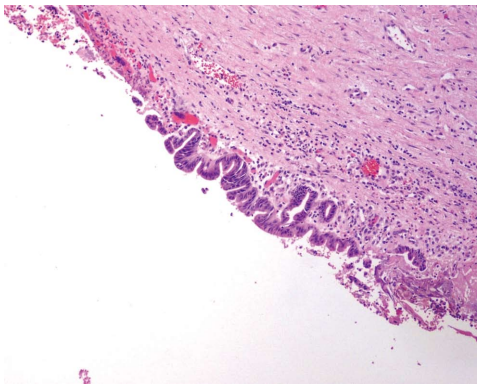

Figure 4 Histology of the intraductal papillary mucinous neoplasm of the bile duct showing papillary proliferation.

resonance imaging, possibly due to its cystic nature. In IPMN-Bs, the bile ducts are frequently dilated due to mucin hypersecretion and accumulation. In our case, the hepatic and common bile ducts were dilated mainly due to the large size of the neoplasm obstructing biliary outflow. Dilated peripheral bile ducts in IPMN-B may be mistaken for other biliary duct diseases, including Caroli disease. The tumors were symptomatic in our patient, as she complained of abdominal pain, itching, and jaundice. Although the size of the tumors suggested malignant transformation, the histologic features did not suggest malignancy.

In conclusion, we present a unique case of the simultaneous occurrence of a MCN and an IPMN-B in the liver that could be radically resected.

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CASE REPORT

Primary rectal squamous cell carcinoma treated with surgery and radiotherapy

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Abstract

Primary squamous cell carcinoma of the rectum is a rare malignancy, and the discrete dual lesions of rectum are even rarer. There is currently no effective and satisfactory treatment for this disease. Here we report a case of an elderly female with bi-primary squamous cell carcinoma of the rectum treated with radical resection and radiotherapy. The patient is still alive 43 mo after the initial curative resection of the tumor. We suggest that surgery as the primary treatment followed by concomitant radiotherapy may be an effective protocol for elderly patients with rectal squamous cell carcinoma.

Key words: Squamous cell carcinoma; Rectum; Primary; Surgery; Radiotherapy

Core tip: Primary squamous cell carcinoma of the rectum is a rare malignancy, and the discrete dual lesions of rectum are even rarer. Our patient is still alive after surgery as the primary treatment followed by concomitant radiotherapy. It was suggested that surgery combined with radiotherapy may be an effective treatment strategy for patients with rectal squamous cell carcinoma, especially for elderly patients who cannot tolerate chemotherapy.

Wang JF, Wang ZX, Xu XX, Wang C, Liu JZ. Primary rectal squamous cell carcinoma treated with surgery and radiotherapy. *World J Gastroenterol* 2014; 20(14): 4106-4109 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4106.htm
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INTRODUCTION

Colorectal squamous cell carcinoma (SCC) is an extremely rare malignancy of the gastrointestinal tract. Since more than 90% of colorectal diseases are adenocarcinomas, very little information is available in the literature about the etiology, prognosis and optimal treatment of this malignancy. It was reported that the survival rate of rectal SCC is by far lower than that of adenocarcinoma[1]. A multidisciplinary approach has been recommended, and surgical resection should be performed in patients with localized disease if possible. Neoadjuvant/adjuvant therapy of rectal cancer can reduce the high risk of locoregional recurrence. In the present study, we report a case of a primary SCC involving two parts of the rectum, which was treated surgically followed by adjuvant radiation.

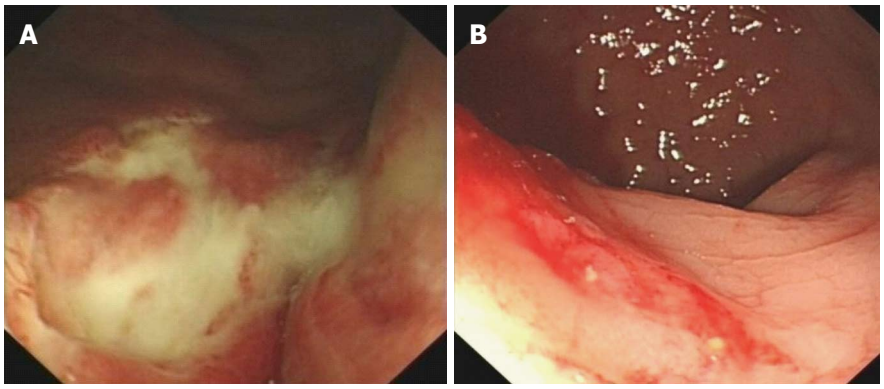

Figure 1 Colonoscopy showing an squamous cell carcinoma of the rectum. The tumors are located about 2 cm (A) and 7 cm (B) from the anal verge, respectively.

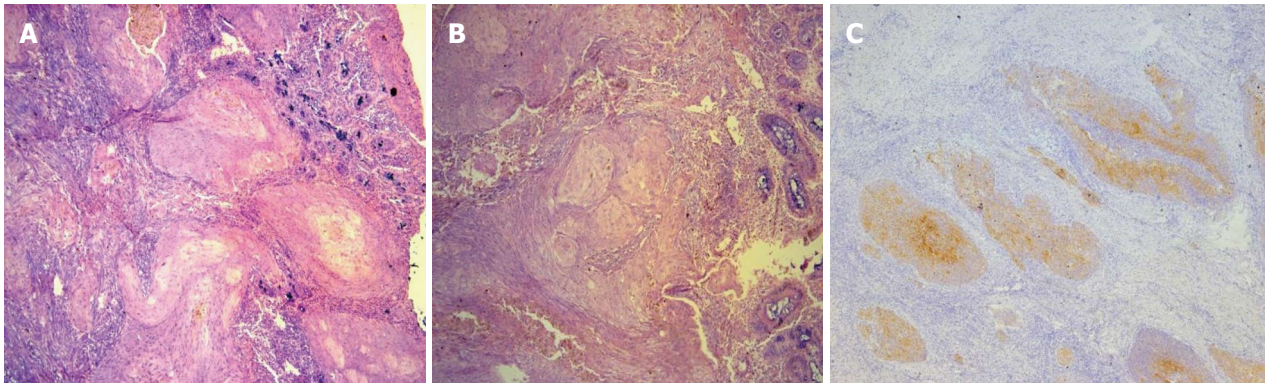

Figure 2 Pathologic examination found a moderately-poorly differentiated squamous cell carcinoma of the rectum. A, B: A high power view of the squamous cell carcinoma of the rectum, showing keratin formation (HE stain, × 100); C: Immunohistochemical analysis of the biopsy of the squamous cell carcinoma of the rectum showing positive CK stain.

CASE REPORT

A 75-year-old female patient was admitted to the surgical department of our center in March 2010 because of perianal discomfort and tenesmus for one and half month. She experienced 2.5 kg weight loss during the past three months. She is a nonsmoker and does not drink. Rectal examination revealed a mass of of 1.5 cm arising 3 cm from the anal edge and another 3.5 cm mass arising 7 cm from the anal edge (Figure 1). Computed tomography (CT) scan of the chest, abdomen and pelvis was negative for distal metastases. The rectal examination was performed using colonoscopy, and biopsies revealed squamous cell carcinoma of the rectum. She then underwent combined abdominal perineal resection of the rectum (Miles operation), and pathologic examination found a moderately-poorly differentiated SCC of the rectum (Figure 2) infiltrating the serosa into the soft tissue of the right pelvic cavity. The margins of the excised tissue were tumor free. Multiple regional lymph nodes seen in the full-thickness excision of the lesion revealed no evidence of invasion. The tumor was found to be T4N0M0 in stage. Postoperatively, the patient was treated with a megavoltage linear accelerator (6 MV) in prone position; and a 2-fields technique (AP-PA fields) was used. The upper border of the radiation field was at the L4/L5 junctions; and the inferior border was at the lower edge of ischial tuberosity including the surgical scar. The left border and the right border included a 1.5-cm margin on the pelvic brim. The radiotherapy doses were specified at the intersection of the central axis of the beams in the pelvis. The dose of 45 Gy was delivered to the pelvis in 25 fractions over 5 wk. A daily dose of 1.8 Gy was given through the AP-PA fields. After the treatment, the patient achieved complete remission confirmed by colonoscopy and computed CT scan of the chest, abdomen and pelvis during the follow-up. At the time of writing this report, the patient had survived 43 mo after treatment, with no clinical evidence of recurrence.

DISCUSSION

Squamous cell carcinoma of the rectum is a relatively rare tumor with a reported incidence of only 0.1%-0.2% of all rectal tumors[2]. This kind of cancer is definitely more common in the anus than in the rectum. The border-line of the two carcinoma types is marked by the 10-12 mm thin transitional layer of the anus, where the cylindrical epithelium changes over to the squamous epithelium[3]. Our case is extremely rare, for the patient presented two lesions in the rectum, both of which were

located > 2 cm from the anal edge.

The pathogenesis of these tumors is still unclear. The most possible assumption is that multipotent basal cells proliferate to squamous cells, and the presence of these undifferentiated basal cells at the base of normal mucosal crypts has been previously demonstrated by Lorenzsonn *et al*[4]. Another possible explanation postulates that ectodermal cells may migrate to the rectum during the embryonic phase and aberrantly proliferate in response to stimuli[5]. Both theories are based on the theory of chronic irritation, *e.g.*, by ulcerative colitis[6], and infection of human papillomavirus (HPV)[7,8], or other viruses such as human immunodeficiency virus[7,9].

The established criteria for a diagnosis of primary SCC of the rectum are as follows[5]: (1) metastases from other sites (*e.g.*, lung SCC) must be ruled out; (2) a squamous cell-lined fistula must not involve the affected bowel; and (3) an SCC of the anus extending to the rectum must be excluded. Immunochemistry is helpful in diagnosing SCC using the markers, including cytokeratins AE1/AE3, 34BE12, CK5 and involucrin in order to differentiate it from other undifferentiated small cell tumors[10].

The optimal treatment for rectal SCC has not been well established due to the rarity of the disease. Traditionally, surgical resection of the affected rectum was considered to be the standard treatment. Local excision is appropriate in selected cases of stage T1 (invasion to the mucosa or submucosa) cancers or possibly stage T2 (invasion to the muscularis propria) lesions. It was reported that T2 lesions particularly require close follow-up, as recurrence after local excision can be as high as 20%[11]. More recently, neoadjuvant radiotherapy (local control of the disease and downstaging) or adjuvant chemo- and/or radio-therapy has been advocated as an alternative primary treatment for SCC with acceptable local control in about 60% of patients[12-16]. Surgery was relegated to the role of salvage therapy for cases that do not respond to radiotherapy[2,3]. However, considering the limited number, the rarity of this condition and the retrospective nature of the series, large randomized prospective trials are needed to identify the role of only chemoradiotherapy. Above all, it is recommended that surgical resection is the treatment of choice, although radiation and chemotherapy can be useful in treating node-positive patients, poorly differentiated cancers and advanced disease. Due to the relatively high risk of locoregional recurrence of rectal cancer of T4N0M0 patients, combined adjuvant therapy after surgery was recommended. However, considering patient's rejection of chemotherapy and the poor toleration of the old age to chemotherapy, we proposed the individualized treatment of radiotherapy after local excision, which yielded a complete response. The patient remained disease-free for 43 mo after treatment. The therapeutic approach with surgery as the primary treatment followed by combined radiotherapy, may be necessary in order to improve the survival and prognosis of patients with local lesions, especially for those of old age.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical staff for their help in the procurement of tumor tissue samples. We also thank the pathologists from the Department of Pathology for the histopathological assessment of the tumor tissues.

COMMENTS

Case characteristics

A 75-year-old female with one and a half month history of perianal discomfort and tenesmus.

Clinical diagnosis

A 2.5 kg weight loss occurred during the past three months and a tumor was found in digital rectal examination.

Differential diagnosis

Colon cancer, rectum adenocarcinoma, colorectal polyps.

Laboratory diagnosis

Carcinoembryonic antigen 2.67 μg/L; squamous cell carcinoma (SCC) 13.9 μg/L; blood routine, liver and kidney function tests were within normal limits.

Imaging diagnosis

Rectal examination revealed a mass of 1.5 cm arising 3 cm from the anal edge and another 3.5 cm mass arising 7 cm from the anal edge.

Pathological diagnosis

Pathology of the surgically resected tissues revealed a squamous cell carcinoma.

Treatment

The patient was treated with combined abdominal-perineal resection of the rectum followed by radiotherapy of totally 4500 cGy in fractions to pelvis.

Related reports

Colorectal SCC is an extremely rare malignancy of the gastrointestinal tract, and very little information is available in the literature about the etiology, prognosis and optimal treatment of this malignancy.

Experience and lessons

Colorectal SCC is a very rare disease and selection of proper treatment is a complex process. However, surgical resection and adjuvant radiotherapy can be considered as a better treatment strategy for patients with early colorectal SCC, especially for those of old age who cannot tolerate the side effect of chemotherapy.

Peer review

In this work, authors reported a case of a 75-year old female patient with bi-primary squamous cell carcinoma. The patient was treated with a curative surgery, followed by radiotherapy with a radiation dose of 45 Gy. The study reported that the patient is no-evidence-disease at the time of writing, 43 months after treatment. Generally, the work presents an interesting case. However, there are several weaknesses to be addressed.

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CASE REPORT

Prolonged small vessel vasculitis with colon mucosal inflammation as first manifestations of Behçet's disease

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Abstract

Behçet's disease is a chronic, relapsing, systemic vasculitis of unknown aetiology. Patients present manifestations of gastrointestinal complications, including mouth lesions, small and large intestinal lesions, and vascular lesions in the abdomen. In some cases, the intestinal ulcers of patients with Behçet's disease are indistinguishable from those of Crohn's disease, tuberculosis, vasculitis and other diseases. In this article, we present a case of atypical Behçet's disease with a complicated medical history and multisystem damage, for the purpose of better management of this disease.

Key words: Behçet's disease; Vasculitis; Intestinal ulcers

Core tip: Behçet's disease is systemic inflammatory vasculitis of unknown aetiology. Its clinical manifestation of gastrointestinal complications varies greatly. Diagnosis is mainly based on typical clinical findings: no specific serum markers or pathological features are available. However, in clinical practice, it is often challenging to make a prompt and correct diagnosis when gastroenteropathy is presented as the initial or predominant manifestation in Behçet's disease patients. The disease is sometimes misdiagnosed as inflammatory bowel disease or other disorders. We present a case of atypical Behçet's disease with a complicated medical history and multisystem damage, for the purpose of better management of this disease.

Yang XN, Ye ZS, Fan YY, Hu YQ. Prolonged small vessel vasculitis with colon mucosal inflammation as first manifestations of Behçet's disease. *World J Gastroenterol* 2014; 20(14): 4110-4114 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/4110.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.4110

INTRODUCTION

Behçet's disease is systemic inflammatory vasculitis of unknown aetiology, characterized by a relapsing episode of oral aphthous ulcers, genital ulcers, cutaneous and ocular lesions, and other manifestations, including vascular, neurological, and gastrointestinal involvement[1].

The clinical manifestation of gastrointestinal complications also varies greatly, from mild symptoms to life-threatening complications, including perforation, infarction and massive bleeding resulting from vasculitis and/or thrombosis[2]. Prompt treatment with corticosteroids plus immunosuppressive agents, rather than surgery, can alleviate the clinical symptoms and improve the prognosis of Behçet's disease patients. The diagnosis of Behçet's disease is mainly based on typical clinical findings: no specific serum markers or pathological features are available. However, in clinical practice, it is often challenging to make a prompt and correct diagnosis when gastroenteropathy is presented as the initial or predomi-

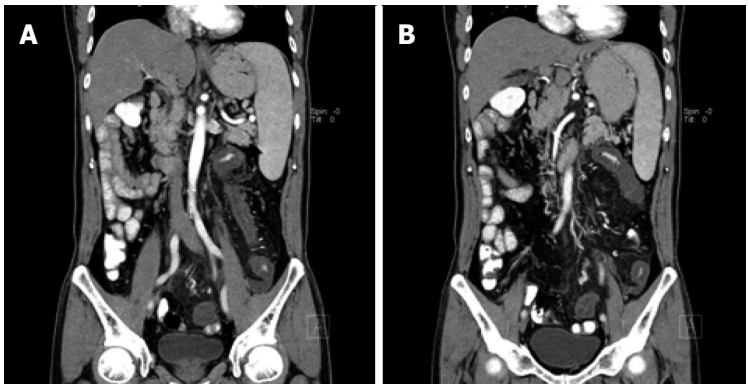

Figure 1 Magnetic resonance imaging of the abdomen and pelvic showed a stenosis of the mesenteric inferior artery branch (A) and thickening of the mesenteric inferior artery branch walls (B). The image shows a diffuse lesion of the left colon suggesting ischaemic injury.

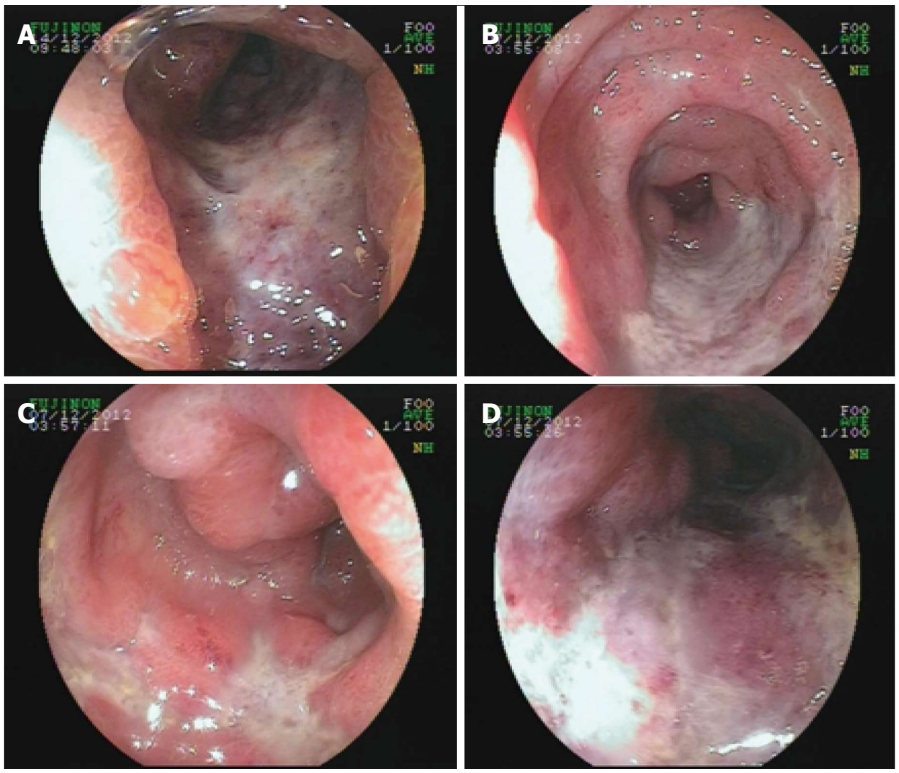

Figure 2 Colonoscopy showed a deep ulcer lesion and granulated tissue of the sigmoid colon and rectum. Lumenal stenosis is obvious.

nant manifestation of Behçet's disease. The disease is sometimes misdiagnosed as inflammatory bowel disease (IBD) or other disorders. In this paper, we present a case of atypical Behçet's disease with a complicated medical history and multisystem damage, for the purpose of better management of this disease.

CASE REPORT

A 56-year-old Asian male presented to the department of gastroenterology complaining of acute-onset bloody diarrhoea for 2 h and generally feeling unwell, with abdominal pain accompanied by mushy stool for the last 3 mo. He described symptoms including pressure in the belly or abdominal pain, stool with mucous (over 10 times each day), progressive fatigue and dizziness. He had no fever, sweating, nausea, vomiting, rash, mucosal lesions, musculoskeletal problem or oral ulcer. Approximately 2 mo before admission, when the abdominal mass appeared, he was evaluated at the hospital. Laboratory findings were as follows: increased erythrocyte sedimentation rate (22 mm/h); C-reactive protein (132 mg/dL); D-dimer (1972 mg/L); and CA19-9 (130.7 U/mL). Abnormal protein patterns in blood serum were found by serum protein electrophoresis (albumin 58%, α-globulin 3.6%, β-globulin 18.7%). Additional tests, including anti-nuclear antibodies (ANA), tuberculosis antibody and antineutrophil cytoplasmic antibodies (ANCA), were within normal limits and the stool bacterial culture was negative. Abdominal magnetic resonance imaging revealed stenosis of the mesenteric inferior artery branch and thickening of the mesenteric inferior artery branch walls (Figure 1). A diffuse lesion of the left colon associated with inflammatory changes was also observed. A diagnosis of ischaemic bowel disease was made. He went to another hospital where colonoscopy was performed, which was suggestive of stenosis and stiffness of the left colon (Figure 2). Digital subtraction angiography of the superior mesenteric artery confirmed vascular malformation (Figure 3). An ultrasound scan of the scrotal sac showed changes con-

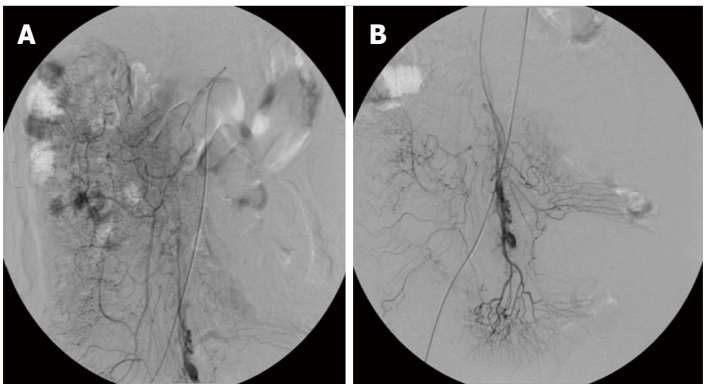

Figure 3 Digital subtraction angiography showed abnormalities in the architecture of the superior mesenteric artery including chaotic distribution (A) and pooling of contrast material (B).

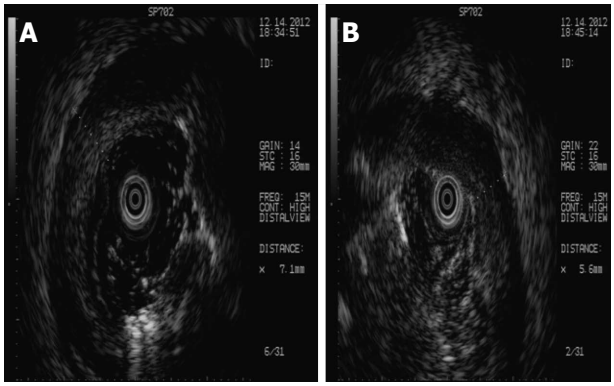

Figure 4 Endoscopic ultrasonography showed ulcers and necrosis in the depth of the mucosa or submucosa (A) and circumscribed mucosal thickening (B). The image shows a characteristic change of chronic nonspecific inflammation, but not malignant lymphoma.

sistent with hydrocele of the tunica vaginalis and epididymo-orchitis. He was given various diagnoses including congenital mesenteric vascular malformation, ischaemic bowel disease and herpes zoster. The patient was treated empirically to improve his circulation and with antivirus therapy, followed by treatment for nutritional support, which achieved remission of his symptoms. Two hours before admission, with acute-onset bloody diarrhoea, the patient came to our hospital and was admitted.

The patient had a history of surgery 6 years previously. On that occasion, he had complained of abdominal pain and haemafaecia. Acute intestinal obstruction was diagnosed; therefore, a partial resection of the ileum and anastomosis were performed. He had also received surgery for an ankle cyst and wrist tenosynovitis a few years ago. There was a family history of immune system diseases. His daughter has Mediterranean anaemia and Hashimoto's thyroiditis.

Physical examination revealed a male in mild distress. Vital signs were normal and the lungs were clear to auscultation. A cardiac examination revealed normal heart sounds and no murmurs. The abdomen was soft. Other significant physical examination findings included a healed surgical scar at the site of previous abdominal operation on the midline, and on the left wrist and ankle for operations for ankle cyst and wrist tenosynovitis, respectively. There was a mass (3 cm × 4 cm) on deep palpation in the right lower quadrant of the abdomen. There was no lymph node enlargement and no oral, respiratory, cardiovascular, abdominal, neurological, musculoskeletal or genital abnormalities. The remainder of the examination was normal.

Initial studies included a general laboratory investigation, chest X-ray and electrocardiography (ECG). A blood test was normal except for platelet counts (57 × 10^9/L). Urinalysis showed a positive occult blood test and positive urobilinogen. Routine stool analysis found pus cells (0-1/Hp), red blood cells (1/Hp), and a defecate occult blood test was positive. Serological tests were performed and showed an increased erythrocyte sedimentation rate (48 mm/h), C-reactive protein (12.2 mg/dL), and hyperbilirubinemia (TBIL: 23.4 μmol/L, DBIL: 7.2 μmol/L, IBIL: 16.2 μmol/L). Additional tests, including ANA, ENA, and ANCA antibodies, complement levels, human immunodeficiency virus antibodies, and hepatitis B and C serology, were within normal limits. The results of radiography of the chest and ECG were normal.

His bloody diarrhoea had diminished the following day, whereas his abdominal pain remained. Considering the history of recurrence of bloody diarrhoea, the colonoscopy was repeated. The endoscopic features, such as deep ulcer lesion, granulated tissue and lumenal stenosis in the sigmoid colon and rectum, raised suspicion of IBD. At this point, a differential diagnosis of intestinal ulcerations including infectious causes such as herpes simplex virus (HSV), tuberculosis, and acute HIV infection and non-infectious causes such as lymphoma, IBD, chronic deep tissue infection, occult solid organ malignancy, vasculitis, developing connective tissue disease, and Behçet's disease was considered. Further investigation included ultrasound colonoscopy (Figure 4), which did not reveal any focal uptake. Sigmoid colon mucosal punch biopsy was performed, which demonstrated formation of granulated tissue, lymphoid hyperplasia, and infiltration of plasma cells, neutrophils, and monocytes into the mucosa. Increased blood vessels with hyaline degeneration and thickening vessel walls were observed. Lymphocytes surrounded the wall of the submucosal venules, especially in the deeper parts. Surgical pathological section of the partial resection of the ileum and anastomosis 6 years ago was studied again and the findings were similar to the present ones, which indicated vascular disruption and damage by neutrophil infiltration. These findings were consistent with vasculitis (Figure 5).

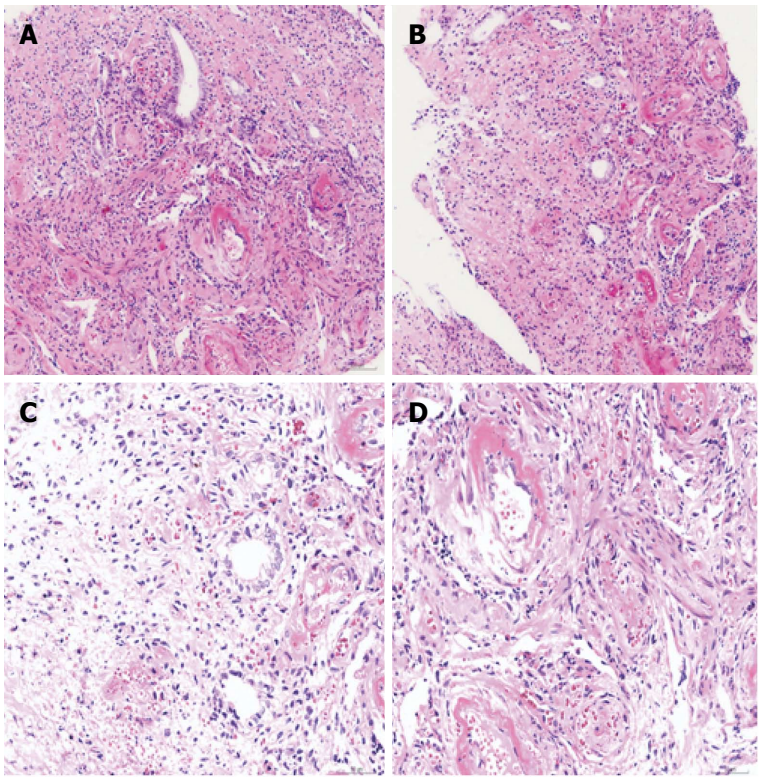

Figure 5 Sigmoid colon mucosal biopsy revealed chronic nonspecific colitis (A, B: HE stain, 100 ×; C, D: HE stain, 200 ×). The image shows formation of granulation tissue, lymphoid hyperplasia, and infiltration of plasma cells, neutrophils, and monocytes into the mucosa. Increased blood vessels with hyaline degeneration and thickening vessel wall are seen. Lymphocytes surround the wall of the submucosal venules, especially in the deeper parts.

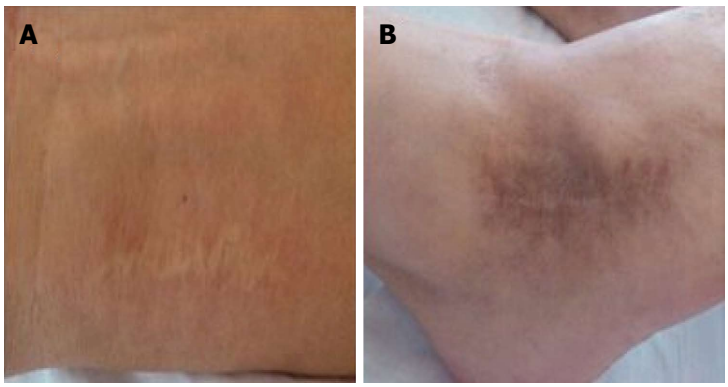

Figure 6 Surgical scar on the left wrist from surgery for tenosynovitis (A) and left ankle cyst (B).

Until this point, progression of the patient's symptoms was consistent with prolonged intestinal ischaemia and vascular damage. Biopsies confirmed the presence of vasculitis and, given the clinical context, he was diagnosed with Behçet's disease, albeit without some typical features, such as oral and genital ulcers. We considered vascular inflammation to be related to vascular immune complex deposits, though small-vessel vasculitis could not be completely ruled out. Asking for the patient's medical history, the wrist and ankle surgery also hinted at the patient having rheumatoid immune disease (Figure 6). The patient was given peroral methylprednisolone 40 mg and leflunomide 10 mg once daily, with quick resolution of his symptoms and disappearance of his abdominal mass.

Two months later, he was hospitalised with acute abdominal pain. A spontaneous sigmoid perforation was diagnosed and sigmoid resection with proximal colostomy was performed. When the use of glucocorticoids was discontinued, recurrent genital ulceration appeared. The patient was finally diagnosed with atypical Behçet's disease and is now being treated with intravenous methylprednisolone at a dose of 20 mg and small doses of gamma globulin. The patient had a complicated hospital stay but responded well to intravenous glucocorticoids and was discharged in a stable condition.

DISCUSSION

Behçet's disease is an inflammatory disorder of unknown cause, characterised by recurrent oral aphthous ulcers, genital ulcers, uveitis and skin lesions. All these common manifestations are self-limiting, except for the ocular attacks. Repeated attacks of uveitis can cause blindness. Behçet's disease is not a chronic, persistent inflammatory disease, but rather comprises recurrent attacks of acute inflammation. Involvement of the gastrointestinal tract, central nervous system and large vessels is less frequent, although it can be life-threatening[3]. Susceptibility to Behçet's disease is strongly associated with the presence of the HLA-B51 allele[3]. Environmental factors, such as

infectious agents, have also been implicated in its pathogenesis.

Vascular injuries, hyperfunction of neutrophils and autoimmune responses are characteristic of Behçet's disease. There are two forms of intestinal involvement: small vessel disease with mucosal inflammation causing ulcers; and large vessel disease resulting in intestinal ischaemia and infarction[4]. Mucosal ulceration is most commonly seen in the ileocaecal region, and was found in about 88% of patients in a recent study[4], usually on the antimesenteric side, followed by involvement of other parts of the colon, but rarely the rectum or anus. The ulcers may be aphthous or, alternatively, deep and round with a punched-out appearance. Longitudinal ulcers are rare.

Behçet's disease does not have any pathognomonic symptoms or laboratory findings; therefore, diagnosis is made on the basis of the criteria proposed by the International Study Group for Behçet's Disease in 2013[5]. According to the criteria, recurrent oral ulceration must be present, as well as at least two of the following: recurrent genital ulceration, eye lesions, skin lesions, and a positive pathergy test. The lesions recur and usually leave scars. The joints most frequently affected are the knees, followed by the wrists, ankles and elbows. Crohn's disease (CD), tuberculosis, vasculitis and other diseases should be excluded before a diagnosis of Behçet's disease is established[6]. Like CD, Behçet's disease manifests as discrete ulcers and discontinuous bowel involvement with relative sparing of the rectum. The two diseases share extraintestinal manifestations, such as uveitis and arthritis. Unlike CD, Behçet's disease is characterised by vasculitis of the small veins and venules, with deep ulcerations, generally without granulomas or cobblestoning. However, both diseases may have chronic nonspecific inflammation with normal intervening mucosa. Perforation is more common in Behçet's disease than in CD, as the latter is characterised by intense fibrosis[7]. Scalloping, ulceronodular patterns and abscess formation are not observed in Behçet's disease[7]. HLA typing may also be helpful in the differential diagnosis.

The choice of treatment depends on the patient's clinical manifestations. Treatment is largely empirical because the heterogeneity of the disease and the unpredictable course with exacerbation and remission make well-controlled studies difficult to conduct. Therefore, there is a lack of evidence-based treatment recommended for the management of Behçet's disease. Agents such as corticosteroids, sulphasalazine, azathioprine, cyclophosphamide, tumour necrosis factor α antagonist, or thalidomide should be tried first before surgery, except in an emergency[8,9].

In conclusion, we describe a patient with gastrointestinal symptoms as primary manifestations of Behçet's disease. Our case highlights the need for clinicians to broaden consideration of differential diagnoses, with particular attention to atypical features of Behçet's disease.

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Acknowledgments

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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