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EDITORIAL

Potential role of NKT regulatory cell ligands for the treatment of immune mediated colitis

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Abstract

Natural killer T lymphocytes (NKT) have been implicated in the regulation of autoimmune processes in both mice and humans. In response to stimuli, this subset of cells rapidly produces large amounts of cytokines thereby provoking immune responses, including protection against autoimmune diseases. NKT cells are present in all lymphoid compartments, but are most abundant in the liver and bone marrow. They are activated by interaction of their T-cell receptor with glycolipids presented by CD1d, a nonpolymorphic, major histocompatibility complex class I-like molecule expressed by antigen presenting cells. Several possible ligands for NKT cells have recently been suggested. β-glucosylceramide, a naturally occurring glycolipid, is a metabolic intermediate in the anabolic and catabolic pathways of complex glycosphingolipids. Like other β-glycolipids, β-glucosylceramide has an immunomodulatory effect in several immune mediated disorders, including immune mediated colitis. Due to the broad impact that NKT cells have on the immune system, there is intense interest in understanding how NKT cells are stimulated and the extent to which NKT cell responses can be controlled. These novel ligands are currently being evaluated in animal models of colitis. Here, we discuss strategies to alter NKT lymphocyte function in various settings and the potential clinical applications of natural glycolipids.

Key words: Natural killer T lymphocyte; Immunomodulatory; Colitis; Inflammatory bowel disease; Ligand

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NKT REGULATORY LYMPHOCYTES

The term 'NKT cells' was first described in 1995[1] and defines a broad subset of mouse T-cells that share some characteristics with natural killer (NK cells), expression of the NK1.1 marker in particular. This is a heterogeneous subset of lymphocytes some of which do not express the NK1.1 marker[2]. NKT cells develop from thymocyte progenitor cells similarly to conventional T-cells. However, unlike conventional T-cells, NKT cells express a T-cell receptor (TCR) that recognizes glycolipids rather than protein antigens[3]. The largest subset of NKT cells expresses a highly restricted TCR comprised of an invariant TCR α chain with a single rearrangement (in mice Vα14-Jα18, and in humans Vα24-Jα18)[4] coupled with TCR β chains with limited heterogeneity due to marked skewing of Vβ gene usage (mostly Vβ8.2 in mice and Vβ11 in humans)[5]. This population, also referred to as invariant NKT cells (iNKT), is highly conserved in most mammals studied to date. iNKT cells are restricted by the major histocompatibility complex (MHC) class I-like molecule CD1d, which is expressed by conventional antigen presenting cells (APCs) including macrophages, dendritic cells, and marginal zone B cells[2].

CD1d-mediated glycolipid presentation to NKT cells is an important aspect of immune regulation. However, as an illustration of NKT complexity, there is a type of NKT-cell that expresses the NK1.1 marker, but is CD1d independent. There are two broad classes of cells that satisfy the criteria of being CD1d dependent NKT cells. For the purposes of this review, we classify these as type I NKT cells, being the Vα14-Jα18 (mouse) or Vα24-Jα18 (human) population, and type II NKT cells, which includes all other CD1d-dependent T cells[6].

The inherent, low-level auto-reactivity of certain specialized immune cell types that have both innate and adaptive characteristics, such as CD1d restricted NKT cells, γδ T cells, and B1 cells, suggests that these cell types may also have the potential to stimulate autoimmunity[2]. Activation of iNKT cells occurs early in a number of microbial infection models in mice, and such activation can lead to reinforcement of the innate immunity and promote subsequent adaptive immunity. Thus, immune responses to certain bacterial, viral, and parasitic infections and tumors can be enhanced whereas autoimmune disease and allograft rejection can be suppressed[5].

THE ROLE OF NKT CELLS IN IMMUNE RESPONSES

NKT cell Th1 and Th2 responses can offset one another;

therefore, polarizing cytokine release toward either one may serve as an important therapeutic tool[7]. These lymphocytes constitutively express cytokine mRNA, and within hours of activation produce large amounts of cytokines such as IFN-γ, TNF, IL-4, and IL-10[5]. NKT cell-mediated regulation of immune responses has been demonstrated to influence a large number of disease states[5]. These cells have received considerable attention in recent years as innate lymphocytes that can modulate T-cell and APC functions in autoimmunity. A potential link between NKT cells and autoimmunity was suggested by the finding that various mouse strains, including non-obese diabetic (NOD) mice that are genetically susceptible to autoimmunity[8,9], have a reduced number and defective function of iNKT cells as compared with non-autoimmune mouse strains[10]. Diminished numbers of NKT cells have been correlated with an increased incidence of autoimmune diseases including systemic lupus erythematosus, scleroderma, type I diabetes, multiple sclerosis, and rheumatoid arthritis[11-16].

The adoptive transfer of NKT cells has ameliorated disease in several immune-mediated animal models, including experimental autoimmune encephalomyelitis[17], immune mediated colitis[18], and graft versus host disease (GVHD)[19]. In addition, NKT lymphocytes play an important role in diverse neoplastic and infectious processes, and as such may serve as a target for potential new immune-therapeutic strategies[20,21]. NKT cells are now known to be a major source of IFN-γ, which is required for early activation of macrophage bactericidal activity[22]. Several studies have demonstrated a role for NKT lymphocytes in anti-tumor immunity[23]. Mouse and human NKT cells were shown to exert cytotoxic activity towards several tumor cell lines[24]. NKT lymphocytes were found to promote tumor rejection in experimental models of tumor immunotherapy by administration of IL-12 or α-GalCer[25]. In a murine hepatocellular carcinoma (HCC) model, NKT cells were shown to have a role in oral immune regulation with HCC lysate and HBV envelope proteins, and in adoptive transfer of dendritic cells pulsed *ex vivo* with the same antigens[20].

LIGANDS FOR NKT REGULATORY CELLS

Through their semi-invariant TCR, NKT cells recognize glycolipids presented in the context of the CD1d molecule[26]. CD1 proteins are a family of molecules that have structural homology to MHC class I molecules, but are unusual in their ability to present glycolipid antigens to T-cells[27]. Because NKT cells can produce cytokines that result in conflicting responses, the possibility exists that the ligand structure can polarize NKT cell responses toward either a Th1 or a Th2 response[28].

Glycosphingolipids, or glycolipids, are a family of both naturally occurring and synthetic molecules composed of a hydrophobic ceramide backbone, N-acylsphingosine, and a hydrophilic head group made of carbohydrates, mono- or oligosaccharides[29]. Enzymatic defects and subsequent accumulation of certain glycolipids can lead to "storage" diseases such as metachromatic leukodystrophy, Gaucher's or Fabry's disease[30]. Patients with Gaucher's disease have altered humoral and cellular immune profiles[31] and increased peripheral blood NKT lymphocytes[32]. In the context of stimulatory glycolipids, an understanding of how glycolipid structure affects cytokine release profiles is essential.

α-galactosylceramide (α-GalCer) was originally discovered during a screen for reagents derived from the marine sponge *Agelas mauritianus* that prevented tumor metastasis in mice[33]. KRN7000, the synthetic α-GalCer analogue, is a high-affinity ligand for the CD1d molecule[34]. *In vivo* administration of α-GalCer to mice or humans results in rapid and robust cytokine secretion by iNKT cells, followed by the activation of a variety of cell types of the innate and adaptive immune systems[35].

OCH is a truncated analogue of α-GalCer in which the sphingosine chain has been shortened from 18 to 9 carbons. Following its administration to mice, the early production of IL-4 by NKT cells remained intact while the bulk of IFN-γ, mostly derived from NK cells, was lost, leading to a Th-2 biased response[36]. The ratio of IL-4 to IFN-γ released by NKT cells is influenced by the length of the lipid chain; shorter chain lengths increase this ratio[3]. Administration of α-C-GalCer leads to a strong Th-1 biased response with sustained IFN-γ levels for several days compared to the 24-h response induced by α-GalCer[37]. Treatment with α-C-GalCer was more potent than α-GalCer in mouse models of malaria and malignant tumors, while treatment with OCH was more efficacious than α-GalCer in the Th-1 mediated autoimmune disease models of encephalomyelitis and colitis[38].

Activation of NKT cells *via* α-GalCer has been shown to affect numerous models of malignancy, infection, and autoimmune disease[3]. In models with strong NKT cell involvement, such as in type I diabetes-prone NOD mice, activation of NKT cells with α-GalCer delayed disease induction and prevented its recurrence[39,40]. On the other hand, treatment with α-GalCer can cause disease exacerbation, an effect noted mainly in models where these molecules play a "pathogenic" role such as in the F1 mouse model of lupus nephritis (NZB × NZW)[41], or the apolipoprotein E knockout mouse model of atherosclerosis[42,43]. Despite their promising effects in diverse disease situations, the clinical use of α-glycolipids has been limited by their side effects, mainly hepatotoxicity[44,45].

NATURAL LIGANDS FOR NKT CELLS

The discovery of the marine sponge-derived glycolipids as ligands for NKT cells led to studies looking for possible natural ligands. These natural antigens can be separated into two groups: (1) antigens that are produced by the host (endogenous antigens), and (2) antigens from foreign pathogens (exogenous antigens). The strongest evidence for the presence of an endogenous antigen is that positive selection of NKT cells in the thymus requires presentation of an antigen recognized by the TCR[3]. The best evidence for the presence of exogenous antigens is that antigen presentation proteins related to CD1d have been characterized as presenters of microbial glycolipids, and it was speculated that NKT cells might survey for the

presence of infectious agents[46-48].

Given the auto-reactivity of the NKT TCR to CD1d and the limited diversity of TCRs that NKT cells express, it is generally accepted that a single, or set of closely related, autologous glycolipid ligands are responsible for the activation of these cells. These endogenous ligands have yet to be identified. Recently, the lysosomal glycolipid, isoglobotrihexosylceramide (iGb3) has been proposed as a natural ligand for NKT cells[49]. This beta structured-glycolipid, in its natural or synthetic forms, has the ability to activate most human or mouse NKT cells *in vitro*. Impaired generation of lysosomal iGb3 in mice lacking β-hexosaminidase *b* resulted in severe NKT cell deficiency, suggesting a role for iGb3 in murine NKT cell development[49]. Recently, some NKT cell activating antigens of microbial origin have been found[50]. NKT cells have been found to play a role in controlling infection by organisms such as *Mycobacterium tuberculosis* where NKT cells predominate in the anti-mycobacterial granulomatous reaction[51,52], *Plasmodium bergehi*, *Listeria monocytogenes*[53], *Ehrlichia muris*, and *Sphingomonas capsulata*[54].

At least two mechanisms have been proposed for NKT cell activation. The first is "enhanced auto reactivity", where APC recognition of microbial antigen results in IL-12 mediated APC-NKT cell activation. The second is a CD1d presented microbial glycolipid that triggers iNKT cells through TCR recognition[2,3]. There has been some success in identifying specific microbial glycolipid ligands of CD1d that can activate NKT cells, most notably, α-glycuronosylceramides (α-galacturonosyl and α-glucuronosylceramide) derived from the lipopolysaccharide-negative *Sphingomonas* bacteria cell wall[55]. These α-glycuronosylceramides are of specific significance because they share structural homology with α-GalCer. Other examples include the CD1-restricted presentation of *Plasmodium berghei* sporozoite-derived GPI anchor that stimulates NKT-cell-mediated B-cell activation and antibody production[56], and the phosphatidylinositol tetramannoside (PIM4) produced by *Mycobacterium bovis*[57]. These activities suggest a role for NKT cells in the innate response against pathogens that do not activate classical pattern-recognition receptors, such as Toll-like receptor 4.

β-GLYCOLIPIDS AS NKT LIGANDS

Recent studies have shown that different glycolipids preferentially target different organelles. Because different isoforms of CD1 localize to different subcellular compartments, they allow APCs to present a variety of glycolipid antigens that enter the cell by different pathways and are targeted to different locations[58]. β-glycolipids are naturally occurring intermediates in the anabolic and catabolic pathways of complex glycosphingolipids and are found in cell membranes[59]. Past studies have suggested that β-glycolipids do not possess stimulatory properties on NKT cells[59]. However, recent data have suggested that these compounds may have an important NKT cell mediated immune modulatory effect. β-glucosylceramide (GC) is a beta glycolipid that is degraded into ceramide by glucocerebrosidase. CD1d-bound GC does not stimulate NKT cells directly[60]. β-glycolipids may inhibit NKT activation and even block the stimulatory effect of α-GalCer on these cells. Glucosylceramide-synthase deficiency leads to defective ligand presentation by CD1d, with secondary inhibition of NKT cell activation[60]. *In vitro*, administration of GC led to a 42% decrease in NKT cell proliferation in the presence of DCs, but not in their absence[61]. Additional naturally occurring β-glycolipids such as β-lactosylceramide (LC) and β-galactosylceramide (GLC) are being tested for their immunomodulatory effects (unpublished data).

Administration of β-glycolipids in several Th1 mediated disease models such as auto-immune hepatitis, metabolic syndrome, and acute GVHD, alleviated the disease while inducing a Th2 cytokine profile[61-63]. In a murine model of concanavalin A-induced hepatitis, administration of GC led to significant amelioration of liver damage[61]. This beneficial effect was associated with a 20% decrease in intrahepatic NKT lymphocytes, a significant lowering of serum IFN-γ levels, and decreased STAT-1 and STAT-6 expression. The administration of GC to leptin-deficient ob/ob mice, an NKT dependent model, significantly improved the metabolic alterations[62]. Liver fat content was reduced significantly in both MRI and histological examinations. In addition, treated mice achieved near-normalization of glucose tolerance and decreased serum triglyceride levels. These effects have been associated with a marked increase of the peripheral/intrahepatic NKT cell ratio. In a semi-allogeneic model of acute GVHD, GC-treated mice manifested a significant decrease in skin, bowel, and liver GVHD manifestations[64]. The beneficial effect of GC was associated with decreased IFN-γ and increased serum IL-4 levels, as well as a significant increase in the intrahepatic to peripheral NKT lymphocyte ratio and in intrahepatic $CD8^+$ lymphocyte trapping[64]. In contrast, in Th2 mediated models of disease, administration of β-glycolipids also led to NKT mediated disease alleviation associated with an opposite Th1 immune shift. In a murine model of hepatocellular carcinoma, GC led to improved survival rates and a decreased tumor volume[63]. These effects have been associated with an 11-fold increase in intrahepatic NKT lymphocyte number. Taken together, these results suggest that certain β-glycolipids may serve as a "fine tuners" for NKT lymphocyte-mediated immune responses and may have a beneficial effect in seemingly opposing disease models.

NKT CELLS IN INFLAMMATORY BOWEL DISEASE

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract that are associated with an imbalance between Th1 pro-inflammatory and Th2 anti-inflammatory subtypes of immune responses. The abundance of CD1d-positive cells in the human intestine suggests a role for these cells in chronic inflammatory disorders of the bowel. NKT cells have been proposed to make both protective and pathogenic contributions to IBD[65]. Ulcerative colitis (UC) is a subtype of IBD that is limited to the superficial

layers of the colon and is dominated by the production of Th2 cytokines. Studies have shown that classical (type I) CD1d-restricted NKT cells contribute to a murine model for UC[66,67]. NKT cells exerted protective effects against DSS colitis, a model for intestinal inflammation that primarily targets mucosal macrophages. In this model, administration of α-GalCer and adoptive transfer of NKT cells resulted in reduction of inflammation.

The role of NKT cells in chronic bowel inflammation is complex. They can play either a protective or a pathogenic role in intestinal inflammation, depending on the type of inflammatory process and the antigen presented in the gut. NKT cells support a pro-inflammatory immune response in TNBS-colitis, a Th1 model. Thus, depletion of NKT cells results in alleviation of the disease[68], effects which were mediated by altered intrahepatic $CD8^+$ trapping and that increased INF-γ producing lymphocytes[69]. Feeding colitis-extracted proteins (CEP) to mice with TNBS-induced colitis induces oral tolerance and alleviates TNBS-induced colitis[70]; NKT depletion prevents oral tolerance induction18. Adoptive transfer of *ex vivo* CEP-pulsed NKT cells also alleviated colitis[69]. NKT cells exerted protective effects against DSS colitis, a model for intestinal inflammation (Th2 model) that primarily targets mucosal macrophages. In this model, administration of α-GalCer and adoptive transfer of NKT cells reduced inflammation. In contrast, oxazlone-colitis could not be induced in animals lacking NKT[71]. Several studies proposed a role for NKT cell activation in IBD patients. Expression of CD1d is higher in the epithelia of the affected terminal ilea of CD patients and in the affected cecum of UC patients, which may lead to recruitment of proinflammatory CD1d-reactive cells from the periphery, resulting in mucosal destruction[72]. However, a more recent report suggested that, in contrast to normal colon surface epithelium, epithelial cells derived from UC or CD patients do not express CD1d[73]. The diminished expression of CD1d was suggested as a possible mechanism for impaired regulatory NKT cell function in IBD. Taken together, these data suggest a complex role for NKT cells in chronic inflammatory disorders of the bowel, which may involve various factors in the immune microenvironment.

EFFECT OF NKT LIGANDS IN ANIMAL MODELS OF IMMUNE MEDIATED COLITIS

Experimental colitis induced by intracolonic installation of TNBS, is associated with a Th-1 immune response as evidenced by increased IFN-γ secretion, decreased IL-10 and IL-4 secretion, and reduction in the intrahepatic $CD8^+$ trapping. These effects were hypothesized to be mediated by regulatory NKT cells[69]. Several glycolipid derivatives have been shown to alleviate hapten mediated colitis. OCH, and α-Gal-Cer analogue with truncated sphingosine chain, attenuates colonic inflammation as defined by body weights and histological injury[38]. The protective effects could not be observed in Vα14 NKT cell-deficient mice, further evidence of an NKT role in the pathogenesis of colitis. The immunomodulatory effect of several β-glycolipids, including GC (glucosylceramide), LC (lactosylceramide), GLC (galactosylceramide), and IGL (GC + LC), was shown to be associated with increased survival and significant alleviation of colitis with improvement in the macroscopic and microscopic scores[63]. Administration of GC alleviated immune mediated experimental colitis, improving both the macroscopic and microscopic scores. The beneficial effects of GC were associated with an increased peripheral/intrahepatic CD4/CD8 lymphocyte ratio and a Th2 immune shift.

In summary, NKT cells may make both protective and pathogenic contributions to IBD[65]. Studies show that these cells are involved in the maintenance of mucosal homeostasis. On the other hand, this subset of cells plays a pathogenic role in human ulcerative colitis. Similar contrasting data have been generated in murine models of IBD[65]. Whether the apparent differences in NKT response patterns depends on variations in NKT ligands and/or on the presence of specific subsets of mucosal NKT cells remains to be elucidated. Further studies that determine the subset of NKT cells and the specific ligands involved in these disorders may facilitate the development of novel therapies for IBD.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Rectal cancer treatment: Improving the picture

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Abstract

Multidisciplinary approach for rectal cancer treatment is currently well defined. Nevertheless, new and promising advances are enriching the portrait. Since the US NIH Consensus in the early 90's some new characters have been added. A bird's-eye view along the last decade shows the main milestones in the development of rectal cancer treatment protocols. New drugs, in combination with radiotherapy are being tested to increase response and tumor control outcomes. However, therapeutic intensity is often associated with toxicity. Thus, innovative strategies are needed to create a better-balanced therapeutic ratio. Molecular targeted therapies and improved technology for delivering radiotherapy respond to the need for accuracy and precision in rectal cancer treatment.

Key words: Rectal cancer; Chemoradiotherapy; Intensity-modulated radiation therapy; Molecular targeted therapy

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WHERE WE ARE: INTRODUCING THE CHARACTERS

Since the early 90's, radical surgery and fluoropirimidine-based chemoradiotherapy (CHRT) are the gold standards of treatment for locally advanced rectal cancer. Studies conducted by the Gastrointestinal Tumor Study Group[1,2] and the North Central Cancer Treatment Group[3] concluded that the combination of postoperative chemotherapy with radiotherapy improved local tumor control and survival in stage Ⅱ and Ⅲ rectal cancer relative to surgery alone.

Although currently the big picture mostly remains, some of the characters of the puzzle have changed. The main milestones in this development began with the improvement of the surgical technique, total mesorectal excision (TME). TME became the choice surgical procedure, with a relevant increase in local control. Actually, at some point it was thought that TME could make radiotherapy (RT) unnecessary. Nevertheless, a randomized study soon followed showing the maintained benefit of RT despite an excellent surgery, at least in terms of local control[4], outcomes that even are improving with longer follow-up.

The second landmark was to move the CHRT segment before the surgery. Initially, preoperative radiotherapy was found to improve overall survival as compared with surgery alone[5,6]. In the last decade, the dominant tendency in the therapeutic development of rectal cancer, both in Europe and North America, has been the use of preoperative radiotherapy with conventional protracted fractionation (45-50 Gy in daily fractions of 1.8-2 Gy during 5-6 wk) with concurrent chemotherapy followed by surgery at 4-8 wk. Extensive experience with preoperative CHRT showed feasibility and promising results in terms of down staging, sphincter preservation and disease control and survival parameters as interesting elements of analysis, with an acceptable toxicity profile. The most frequently used chemotherapy agent in this clinical context is 5-fluorouracil (5-FU, i.v.)[7-13]. More recently, the only phase Ⅲ trial concluded comparing pre- *vs* post-operative CHRT, demonstrated better tolerance, sphincter-saving surgical procedures and local control with preoperative CHRT[14].

Preoperative radiotherapy alone (no chemotherapy) and delayed surgery reported down staging rates of 18%[15,16]. However, the prolonged administration of CH-RT achieves down staging figures of around 65%[7-11,17]. Additionally, induction of tumor down staging improves the probability of a complete resection and sphincter-preserving surgery[11,13,18-20].

Complete pathologic response (pCR) rates range from 8% to 27% using i.v. 5-FU with preoperative irradiation[7,10,11,14,21]. In studies of postoperative 5-FU-based CHRT, severe acute toxicity ranges from 24%-40%[1,14,22,23]. However, in Phase Ⅱ studies of preoperative CH-RT, Grade 3-4 acute toxicity occurs in 15%-28% of patients[7,11,13,14,20].

Regarding tumor control and survival, published series

Table 1 Novel chemoradiation combinations

	Chemotherapy		RT (Gy)	GI grade 3-4 toxicity (%)	DS (%)	pCR (%)
	Capecitabine (mg/m^2 bid)					
Kim *et al*	825 d 1-14 and 22 - 35		50.4	-	84	31
De Paoli *et al*	825 bid continuous		50.4	-	57	24
	5-FU (mg/m^2 CI)	CPT-11 (mg/m^2 weekly)				
Mehta *et al*	200	50	50.4	28	71	37
Klautke *et al*	250	40	50.4	32	76	24
Mohiuddin *et al*	Arm 1: 225	Arm 1: -	HART: 55.2-60	27	78	26
	Arm 2: 225	Arm 2: 50	50-54	37	78	26
Navarro *et al*	225	50	45	14	49	14
	5-FU (mg/m^2 CI)	Oxaliplatin (mg/m^2)				
Ryan *et al*	200	MTD: 60 weekly	50.4	38	-	25
Aschele *et al*	200-225	MTD: 60 weekly	50.4	16	84	28
Turrito *et al*	300	80 wk 1, 3, 5	45	-	65	15
	Capecitabine (mg/m^2 bid)	Oxaliplatin (mg/m^2)				
Rodel *et al*	825 d 1-14 and 22 - 35	50 d 1, 8, 22	50.4	6	55	19
Machiels *et al*	825 bid continuous	50 weekly	45	30	-	14

RT: Radiotherapy; DS: Downstaging; bid:Twice daily; CI: Continuous infusion; HART: Hyperfractionated accelerated radiotherapy; MTD: Maximun-tolerated-dose; GI: Gastrointestinal.

vary in follow-up. Preoperative CHRT in rectal cancer assumes ranges for 5-year local recurrence from 2% to 15%, disease-free survival from 70% to 86%, and overall survival from 60% to 85%[7,9,10,14,18,21,24-26].

In summary, incorporation of TME surgical procedure and 5-FU-based preoperative CHRT have been translated to an improvement in local control, with the additional advantage of more tolerable treatments in terms of acute toxicity and saving-sphincter surgical procedures.

MOVING FORWARD: IMPROVING THE PORTRAIT

The picture is drawn. What is next, more characters or better colors?

Therapeutic intensity is often linked to better response and outcomes. But in oncology more is not always better. Increases in doses or number of therapeutic agents combined together lead to higher rates of toxicity. This situation is especially true in rectal cancer. Moreover, the risk of over-treatment in some patients with rectal cancer is present. One treatment approach for all rectal adjuvant patients may not be warranted. We already know that not every stage Ⅱ-Ⅲ rectal cancer is the same[27]. Prognostic factors have been studyed, both at clinical and at molecular and genetic level. In the near future these signatures should be taken into account. An adequate therapeutic index should be found, with a well-balanced ratio of benefit/toxicity.

Where can we find additional benefit in rectal cancer treatment? On the one hand, despite the improvement in local control with multimodality approaches, the rate of distant metastasis is still high, around 19%-36%[10,14]. On the other hand, growing data demonstrates a relationship between response to preoperative CHRT and survival. A higher grade of tumor regression in the surgical specimen has been associated with increased disease-free survival and overall survival after preoperative CHRT in rectal cancer[10,24,17,28-31]. Thus, achieving higher rates of complete pathologic response, but also major tumor regression, is one of the current goals in the protocols of preoperative CHRT in rectal cancer. Both effects, reduction of distant metastasis and higher tumor regression grade, require the use of more active and effective chemotherapeutics agents, with adequate toxicity profiles when administered with radiotherapy.

Exploring novel CHRT combinations

Oral fluoropyrimidines: Oral fluoropyrimidines have been developed as a therapeutic alternative to i.v., continuous infusion of 5-FU, and have been shown to deliver similar efficacy and tolerability with the additional advantage of offering the convenience of oral chemotherapy (Table 1).

Few studies have investigated the safety and efficacy of tegafur with or without uracile (5-FU pro-drugs) and radiotherapy[32-35]. Down staging rates (54%-68%), pCR (8%-15%), and grade 3-4 toxicity (12%-43%) match quite well with those with i.v. 5-FU. Although follow-up is not as long as in the 5-FU series, outcomes in terms of local control, distant metastasis rate, disease-free survival and overall survival seem to be similar.

Capecitabine is a fluoropyrimidine carbamate active

in several solid tumors. A recent phase Ⅲ trial (X-ACT trial) has demonstrated the equivalence of capecitabine to bolus 5-FU/leucovorin in the adjuvant treatment of colon cancer[36]. Thymidine phosphorylase (TP) is a key enzyme for the metabolism of capecitabine to 5-FU. Some data suggest that tumor tissue shows higher concentrations of TP than normal tissue[37]. This phenomenon would lead to a preferential activation of capecitabine in the tumor tissue, providing a favorable ratio for toxicity and radiosensitization. Preclinical studies have shown that RT might up-regulate the TP expression in tumor cells, resulting in a selective and synergistic effect between RT and capecitabine[38]. Phase I studies have been conducted to determine the maximun-tolerated-dose (MTD) of capecitabine in combination with radiotherapy. The recommended dose for this combination was 825 mg/m^2 bid without break during radiotherapy period (5-6 wk)[39,40]. Two published phase Ⅱ studies have shown that preoperative CHRT with capecitabine appears to be effective in locally advanced, resectable rectal cancer. Encouraging rates of down staging (up to 84%) and pCR (24%-31%) with a favorable safety profile of the combination might warrant the use of capecitabine and RT with other effective new drugs[40-42].

Irinotecan (CPT-11): Irinotecan is an active chemotherapeutic agent in colorectal cancer. The combination of Irinotecan and 5-FU has been approved as first line chemotherapy for patients with metastatic colorectal cancer[41,43,44]. Phase I studies have demonstrated that CPT-11 can be safely administered concomitantly with radiotherapy (MTD: 10 mg/m^2 daily or 50 mg/m^2 weekly)[45]. Several phase Ⅱ studies have determined the efficacy and feasibility of the irinotecan and 5-FU combined-therapy plus radiotherapy in the neo-adjuvant management of rectal cancer. The rates of tumor down staging (49%-78%) and pCR are high (14%-37%) with an acceptable rate of acute severe toxicity (14%-37%)[46-49].

The combination of CPT-11 and Capecitabine with radiotherapy has been studied in recent phase Ⅰ-Ⅱ trials[50,51]. The MTD dose of Capecitabine was 500 mg/m^2 while combining with CPT-11 50 mg/m^2 weekly and 750 mg/m^2 while combining whit CPT-11 40 mg/m^2 weekly. The rate of tumor down staging and pCR were similar with the two schedules (72%-75% and 14%-21%, respectively) and similar with the combination of 5-FU, CPT-11 and radiotherapy.

Oxaliplatin: Oxaliplatin is a novel anti-neoplastic platinum. When combined with 5-FU, oxaliplatin improves overall survival for patients with metastatic colorectal cancer and the rate of progression-free survival for patients with completely resected stage Ⅱ and Ⅲ colon cancer[52,53]. These data encourage combining oxaliplatin and 5-FU in the preoperative setting of rectal cancer management for an improved response. Moreover, oxaliplatin has radiation sensitization properties[54].

Several phase Ⅱ studies have evaluated weekly administration schedules of oxaliplatin and 5-FU and radiotherapy. They have demonstrated that this regimen is feasible with moderate toxicity. The addition of oxaliplatin to standard 5-FU-RT seems to be associated with a promising down staging (65%-84%) and pCR rates (15%-28%)[55-57].

Oxaliplatin has been combined with Capecitabine in metastatic colorectal disease[58-60]. The combination has been adapted to preoperative CHRT and phase Ⅰ-Ⅱ trials have been published. The studies show that this regimen is active and feasible, with attractive down staging (55%-72%) and pCR rates (14%-28%)[61-63].

RAISING THE BAR: THERAPEUTIC MODULATION

One of the paradigms for loco regional treatment of cancer is anatomic precision. Technical advances in radiation oncology including functional and molecular imaging and intensity-modulated radiation therapy (IMRT) delivery techniques are allowing greater treatment precision and dose escalation. Moreover, cancer is a biologic entity. Treating cancer requires understanding cancer biology which is changing the approach in cancer therapeutics. A number of genetic signatures and molecular pathways involved in cancer have been discovered. Parallel molecular therapeutic development is emerging. Molecular targeted treatments have being combined with conventional anticancer drugs, accordingly with specific tumor biology.

Coming back to loco regional treatment of rectal cancer, IMRT might provide anatomical specificity. Molecular therapies will complement anatomical specificity by targeting biological pathways that are deregulated in individual tumors. Precision is technologically based while accuracy is biologically based[64].

New biological agents: biological modulation

Epidermal growth factor receptor (EGFR) and angiogenesis-related pathways are perhaps the molecular mechanisms best explored in colorectal cancer. Both mechanisms are involved either in colorectal carcinogenesis and tumor growth[65,66], and in radioresistence[67-69]. Thus, novel targeted biologic agents including angiogenesis and EGFR inhibitors hold tremendous promise as RT sensitizers and as systemic therapy in rectal cancer[69-71].

Preliminary reports show feasibility and promising activity combining Bevacizumab with 5-FU and RT. The MTD was determined for Bevacizumad at 5 mg/kg[72]. Additionally, surrogate markers are being investigated suggesting the ability of Bevacizumab to specifically target tumor angiogenesis[72,73].

A recent phase I study combining capecitabine, oxaliplatin and bevacizumab with preoperative RT establishes the MTD to be capecitabine 625 mg/m^2 BID, Oxaliplatin 50 mg/m^2 per week and Bevacizumab 15 mg/kg d 1 and 10 mg/kg d 8 and 22. Down staging was observed in 9/11 patients (82%) and 2/11 (18%) patients achieved pCR and in 2 of 11 only microscopic disease was found in the surgical specimen[74].

C225 (Cetuximab) is a chimeric monoclonal antibody that targets the extracellular domain of epidermal growth

factor receptor (EGFR) with high specificity and affinity[75]. Cetuximab has demonstrated increased responses combined with chemotherapy in metastasic colorectal cancer[76]. The radiosensitization activity of Cetuximab has been broadly explored[77]. Thus, the combination of chemotherapy and RT with C225 is an attractive strategy to be explored.

A pilot study has explored the addition of Cetuximab (250 mg/m^2 per week) to conventional i.v., continuous infusion of 5-FU and RT. Grade 3-4 diarrhea was detected in 10% and acneiform rash in 15%. Pathological complete response was achieved in 12% of patients[78].

Cetuximab has been combined with Capecitabine and RT in rectal cancer. The dose suggested is Capecitabine 825 mg/m^2 bid without interruption during the duration of RT and Cetuximab 250 mg/m^2 weekly. Grade 3 diarrhea was 10%, rectal pain 20%. Ten percent of the evaluated patients achieved pCR[79].

A phase I trail has recently evaluated the combination of Capecitabine, Oxaliplatin and C225 with RT. Doses suggested were for Cetuximab 400 mg/m^2 on d-7, then 6 weekly doses of 250 mg/m^2, for oxaliplatin 50 mg/m^2 d 1, 8, 22 and 29 in combination with capecitabine 1650 mg/m^2 bid d 1-14 and 22-35. Grade 3-4 diarrhea was 15% and grade 3-4 toxicity as skin reaction 7%[80]. The results of the phase Ⅱ study with 31 patients enrolled are coming soon.

Intensity Modulated Radiotherapy in rectal cancer: Rational and preliminary experience

New drugs and biological treatments may enhance global radiotherapy effects improving therapeutic outcomes but acute effects may also be increased. Moreover, a dose-volume relationship has been established between the severity of diarrhea toxicity and the volume of irradiated small bowel at all dose levels in patients treated with preoperative chemoradiation for rectal cancer[81]. The volume of irradiated small bowel thresholds to predict acute gastrointestinal toxicity is unknown although a strong correlation exists between the volume of small bowel receiving 15 Gy (V15) and the degree of acute small bowel toxicity[82].

The development of novel and sophisticated irradiation techniques as intensity modulated radiation therapy (IMRT) represents a spectacular progress in planning and delivering external beam radiation therapy. IMRT generates highly conformal and irregularly shaped dose distribution while reducing dose to adjacent normal tissue structures. IMRT has demonstrated dosimetric superiority over 3D-conformal radiation therapy (3D-CRT) in the majority of tumor sites, including pelvic tumors where the irradiated bowel can be significantly reduced[83].

Researchers at the Royal Marsden Hospital have reported a dosimetric study comparing IMRT *vs* 3D-CRT in five rectal cancer patients. The irradiated bowel volume at 45 Gy and 50 Gy can be reduced with IMRT techniques, which could potentially resulted in marked reductions in acute and chronic bowel toxicity[84]. Tho and colleagues[81] evaluated the role of IMRT in 41 patients with locally advanced rectal cancer treated with preoperative 5FU CHRT. The results showed that IMRT provided dosimetric and radiobiological modeling benefits by reducing the dose to the small bowel, and the likelihood of late normal tissue complications. A dosimetric comparison of 3D-CRT using pelvic anatomical references, 3D-CRT with more restrictive volumes, and IMRT was explored by our institution in nine patients diagnosed with locally advanced rectal cancer. A number of parameters, such as conformity index in the planning target volume, different dose levels at the planning target volume and organs at risk were calculated and compared between the three plans. Target coverage was similar, but the conformity index was better using IMRT. Irradiation doses at small bowel and bladder were significantly reduced with IMRT planning.

Dosimetric parameters in rectal cancer with IMRT are encouraging. Clinical research looking for acute and late toxicity, tumor response, tumor control and survival is warranted. The rationale for the use of chemo-IMRT in locally advanced rectal cancer is based on the potential decrease of gastrointestinal toxicity while maintaining conventional dose to the primary tumor, draining lymph node regions and presacral region. This capacity to change the gastrointestinal toxicity profile may also allow reducing the number of fractions by increasing fraction size, which ultimately may improve the rate of pCR and cost-effectiveness.

Our institution has carried out a prospective study of preoperative chemo-IMRT in rectal cancer. The treatment protocol includes simultaneous combination of capecitabine and oxaliplatin with three escalating dose levels of IMRT, 37.5 Gy 42.5 Gy and 47.5 Gy in 15, 17 and 19 fractions, respectively[85] Chemotherapy consisted on capecitabine 825 mg/m^2 bid during radiation therapy (resting over the weekend) and oxaliplatin 60 mg/m^2 d 1, 8 and 15. Resection was scheduled 6 wk after termination of chemo-IMRT. Simulation was made with the patient positioned prone and immobilized using a combination of prone head cushion and shell with a mixed foam bag. The patient was CT scanned from the L2 vertebral body to the entire perineum with a slice thickness of 5 mm. The slices were transferred through local network to the treatment planning system. The target volumes and organs at risk (OARs) were delineated on axial CT slices in the Helax-TMS treatment planning system (Nucletron Scandinavia, Uppsala, Sweden) as seen in Figure 1. The gross tumor volume (GTV) was defined as the primary tumor and the suspicious metastasic lymph nodes visualized on the CT scan. The clinical target volume (CTV) included the GTV, the presacral region and the common and internal iliac lymph nodes. Adding a margin of 0.5-1 cm around the CTV generated the planning target volume (PTV). The OARs outlined were the bladder and the small bowel. After the GTV, CTV, PTV and OARs were contoured the edited CT slices were transferred from the Helax-TMS treatment planning system to the inverse planning system (KonRad version 2, Siemens Oncology Care Systems, Heidelberg, Germany). Inverse planning for step-and-shoot treatment was performed using 15 MV photons generated on a Mevatron Primus linear accelerator (Siemens Oncology Care Systems, Concord, USA). Seven coplanar equally spaced fields (gantry angles 0°, 51°, 103°,

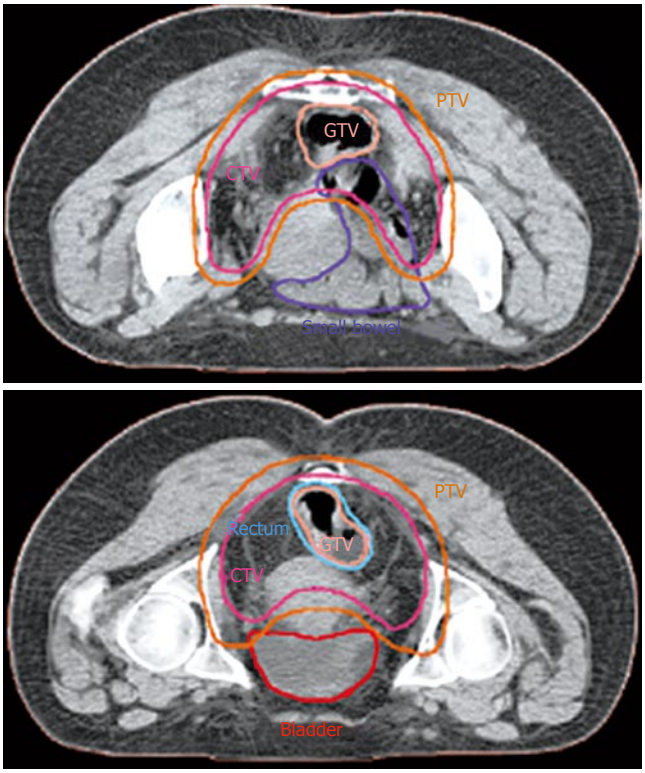

Figure 1 The GTV, PTV and organ at risk (small bowel and bladder) countered on the axial CT slices.

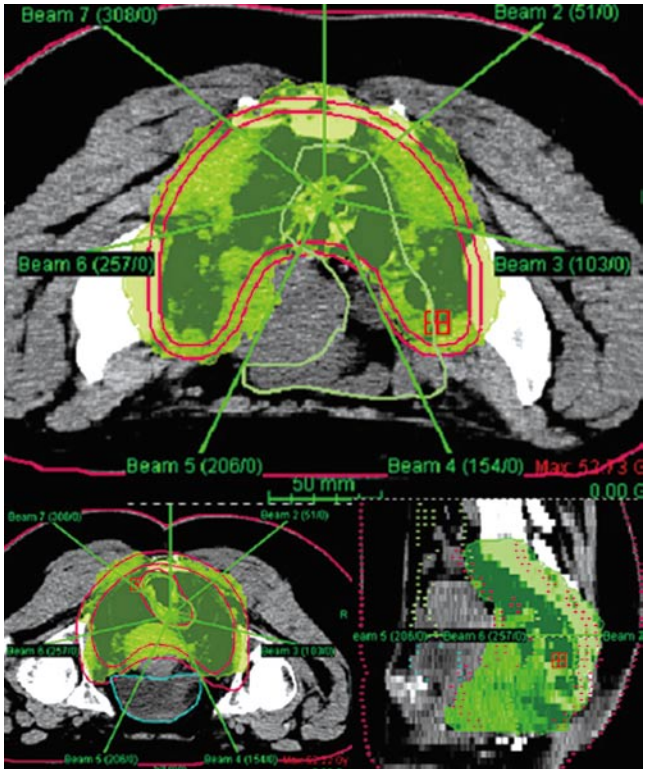

Figure 2 Axial and sagital CT scan images with dose distributions. The 45 Gy isodose surface (green) encompass the GTV and PTV.

154°, 206°, 257° and 308°) were used and the isocenter was placed in the geometric center of the PTV. Figure 2 displays the clinical dosimetry over the patient CT scans.

The first three patients received 37.5 Gy and there were no dose-limiting toxicity (DLT) defined as any grade 3 or 4 gastrointestinal toxicities or grade 4 hematological toxicity. The next three patients received 42.5 Gy without observed DLT and the remaining patients received 47.5 Gy in 19 fractions. Preliminary data show that treatment compliance was 80%, grade 3 adverse events were seen in 21% of the cases, down staging was observed in 52% of patients and pathological response grade 3+ or 4 according to the scale established by Ruo *et al*[86] occurred in 45% of patients.

The use of preoperative IMRT combined with more active systemic chemotherapy provides a major challenge to improve treatment-related toxicity observed with more conventional radiation techniques. Furthermore, the promising favorable pathological response observed with these strategies has the potential to be associated with better loco regional control of disease and may predict better survival.

CONCLUSIONS

Preoperative CHRT followed by TME surgery is the current framework for rectal cancer treatment picture. Further advances with better agents (chemotherapy and molecular targeted therapies) and technology (IMRT) will be translated to improved shapes and colors, enhanced contrast and brightness: response intensity with balanced toxicity.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Moving forward in colorectal cancer research, what proteomics has to tell

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Abstract

Colorectal cancer is the third most common cancer and is highly fatal. During the last several years, research has been primarily based on the study of expression profiles using microarray technology. But now, investigators are putting into practice proteomic analyses of cancer tissues and cells to identify new diagnostic or therapeutic biomarkers for this cancer. Because the proteome reflects the state of a cell, tissue or organism more accurately, much is expected from proteomics to yield better tumor markers for disease diagnosis and therapy monitoring. This review summarizes the most relevant applications of proteomics the biomarker discovery for colorectal cancer.

Key words: Proteomics; Colorectal cancer; Biomarker

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INTRODUCTION

Cancer is not a single disease, but an accumulation of genetic and epigenetic events. It is characterized by uncontrolled growth of cells that can invade and destroy normal tissues. These abnormal cells can also spread through the bloodstream or lymph system to start new tumors in other parts of the body. The disease is a great challenge to clinicians and scientists.

Recent progress in molecular biology has allowed the identification of markers useful for patient management through the identification of genetic alterations and an understanding of chemotherapy molecular targets. Several examples in digestive oncology underline the relevance of molecular biology in clinical research[1].

Colorectal cancer is a common malignancy with an annual incidence of over 945 000 cases worldwide and an annual mortality of 492 000[2]. Surgery is the treatment of choice offering a potential cure. However, 30%-40% of patients have local regionally advanced or metastatic disease on presentation, which cannot be cured by surgery alone[3]. In addition, more than half of patients initially believed to be cured develop recurrence and die of the disease[4].

Advances in genomics and proteomics contribute to our understanding of pathways that control growth, differentiation, and death of cells. In these processes, the identification of candidate disease genes and modifier genes by integrated study of gene expression and metabolite levels is instrumental for future health care. This approach, called systems biology, can recognize early onset of disease and identify new molecular targets for novel drugs in cancer[5].

Proteomics analyzes proteins within a cell or in the corresponding tissue; the proteins of interest are identified, but their function and interactions are not determined. The research provides complete and detailed data about structure, expression, and function of genes, but fails to demonstrate how all the information implicated in the genome is used. In the "post-genomic era," proteomics might be the key to understand systems biology. During the past few years, proteomics has been utilized in many fields of science, medicine, pharmacy, industry and agriculture[6]. In most of the applications proteomics is used to determine expression profiles of proteins in cells and tissues in normal or disease states[7] that are responsible for abnormal cell proliferation. The identification of proteins that are characteristic for cancer development can potentially uncover diagnostic, or prognostic markers, or novel drug targets, and could help understand the mechanisms underlying tumor formation (Figure 1).

Currently, proteomic technology has been used in two areas of cancer research, in early diagnosis and in the treatment of patients, that also includes prediction of response. This technology, when combined with

genomic analysis, may provide more information about the molecular basis of carcinogenesis and the development of more effective anti-cancer therapies. This review focuses on the proteomic studies applied in colorectal cancer.

PROTEOMIC TECHNIQUES IN CANCER RESEARCH

Sample preparation in proteomic

Sample preparation is the most critical step in any proteomics study. This is important because it affects reproducibility as a result of the heterogeneity of proteins derived from cell populations[8]. From the time of sample collection to when proteins are processed for analysis, multiple factors come into play. Mechanical methods, such as surface scrapping and fine needle aspiration, have been used for capturing cancer cells[9]. Calcium depletion and other nonenzymatic methods, such as immunomagnetic separation, have been used to obtain pure populations of cancer cells[10]. An important advancement in sample preparation has been the development of laser capture microdissection (LCM). The LCM system permits obtaining pure populations of cancer cells from frozen, paraffin-embedded, stained, and unstained tissues for molecular analysis. The system is based on visualizing a tissue section via light microscopy and procurement of cells by activating a 7.5-30 micron diameter infrared laser beam which adheres the tissue to a plastic cap. Intact deoxyribonucleic acid, RNA, and protein are then extracted from the adhered tissue which then can be analyzed using conventional methods[11,12]. Protein expression has been compared using 2-D PAGE and differentially expressed proteins identified by mass spectrometry, permitting the discovery of a novel colorectal cancer biomarker[13,14].

Two-dimensional gel electrophoresis and tumor protein detection (2D)

Traditional proteomic studies are based on 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) to compare protein expression patterns from different tissues or cell lines. The first dimension separates proteins by pH, isoelectric focusing, and the second dimension by molecular mass, sodium dodecyl sulfate PAGE. Although, 2-D PAGE has been available for several decades, improvements in this technology have dramatically improved sensitivity, resolution and reproducibility.

The more important application of this technique in disease proteomics is the discovery of proteins which might serve as prognostic biomarkers for survival of cancer patients. A novel application of 2-D PAGE has been in the discovery of circulating autoantibodies in cancer patients. In some cancer patients, there is evidence that a humoral immune response against tumor antigens might be elicited, and this might be used in serum assays of disease progression or in the development of anticancer vaccines.

An advantage of 2-D PAGE is that it has the capacity to resolve and investigate protein, abundance in a single sample and the possibility to directly detect changes in diseased and healthy tissue.

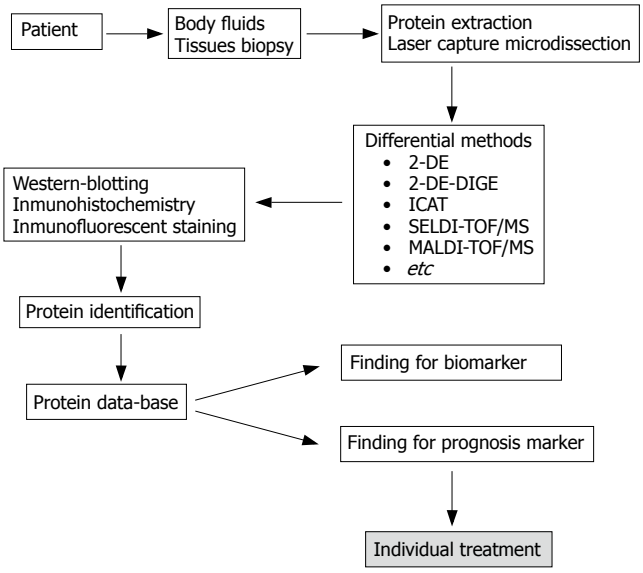

Figure 1 Proteomic differential display methods.

The major disadvantage of 2-D PAGE is that it is laborious and does not resolve highly basic or proteins, smaller than 10 kDa. Because most clinical biomarkers are high large proteins 2-D PAGE is an ideal technology for the study of cancer biomarkers. Therefore, 2-D PAGE, complemented with mass spectrometry, has been used to identify protein changes associated with a variety of human cancers[12].

Two-dimensional difference gel electrophoresis (2D-DIGE)

One of the most recent technical advances in 2-DGE has been multiplexing fluorescent 2D-DIGE[15]. This method directly labels lysine groups in proteins with cyanine (Cy) dyes prior to IEF and can allow for quantitative comparisons between patients and control samples when different fluorescent labels are used for each sample.

The critical aspect of 2D-DIGE technology is the ability to label 2-3 samples with different dyes and then electrophorese all samples on the same 2-D gel. This ability reduces spot pattern variability and the number of gels in an experiment making spot matching much more simple and accurate[16]. The single positive charge of the CyDye replaces the single positive charge present in the lysine at neutral and acidic pH keeping the pI of the protein relatively unchanged. A mass of approximately 500 Da is also added by the CyDye to the labeled protein. The individual protein data from the control and diseased/treatment (Cy5 or Cy3) samples are normalized against the Cy2 dye-labeled sample, Cy5:Cy2 and Cy3:Cy2. These logarithm abundance ratios are then compared between the control and diseased/treatment samples from all the gels using statistical analysis (*t*-test and ANOVA)[17,18]. The principal disadvantage of this technique is that it has a low throughput (three samples per gel) (Figure 2).

Antibody, protein and peptide arrays

Antibody array based measurement technologies have long provided an important tool to detect and manipulate specific biological molecules. While previous uses of

antibodies and related affinity reagents have focused on single targets, recent developments have included multiplexed use of antibodies in arrays, so that many targets can be measured in parallel, sometimes in very small sample volumes. The uses of such arrays are varied and new applications and formats continue to evolve[19].

The experimental features of microarrays have advantages for cancer research. The low sample volumes result in the consumption of small amounts of both precious clinical samples and expensive antibodies. The assays can be run efficiently in parallel, making possible studies on the large populations of samples that are necessary for marker detection and validation. In addition, these assays have good reproducibility, high sensitivity, and quantitative accuracy over large concentration ranges[20]. Antibody and protein arrays are complementary and in some aspects preferable to separation based and mass spectrometry based technologies. Reproducibility and throughput can be higher, and the identities of the considered proteins are known or can be readily characterized. Therefore, specific hypotheses regarding the nature of molecular alterations can be tested, and biologically interpreted[21]. Applications of antibody array methods to cancer research are increasing in scale and effectiveness.

Protein and peptide arrays are effective for probing the interactions of protein and peptides with other antibodies, protein or other molecules. Protein microarrays are an emerging class of nanotechnology for analysing many different proteins simultaneously. Much progress has been made for applications in basic science[22]. These approaches are likely to recapitulate at the protein level the mRNA expression profiling studies by arraying various protein probes on top of specific surfaces, and then determining interactions with specific proteins in complex samples. The most advanced format in this setting is the antibody microarray, where the proteins are specific antibodies printed on solid surfaces.

Protein arrays recently have confirmed the use for probing the abundance of specific proteins in biological samples, this phase call "reverse phase". Protein lysates from cell culture or tissue samples are spotted in microarrays on nitrocellulose membranes. A labeled antibody specific for a particular protein is incubated on a microarray, and quantification of the bound antibody reveals the amount of that protein in each sample[23,24]. Therefore, reverse phase array experiments quantify a single protein in many samples, in contrast to antibody arrays that quantify many proteins in one sample. Numerous demonstrations that this technology uses for profiling proteins in cancer have appeared.

The various methods presented here are complementary with each other and with other proteomic methods, and they may be used together for added benefit as demonstrated in a study of proteins in breast cancer cells using cytokine arrays, reverse phase arrays, and bead-based arrays in conjunction with two-dimensional gels (Figure 3).

TOF-Mass Spectrometry applications in clinical oncology

SELDI-TOF MS is a commonly used non-gel based method. The technique combines protein separation directly with presentation to the mass spectrometer. Various types

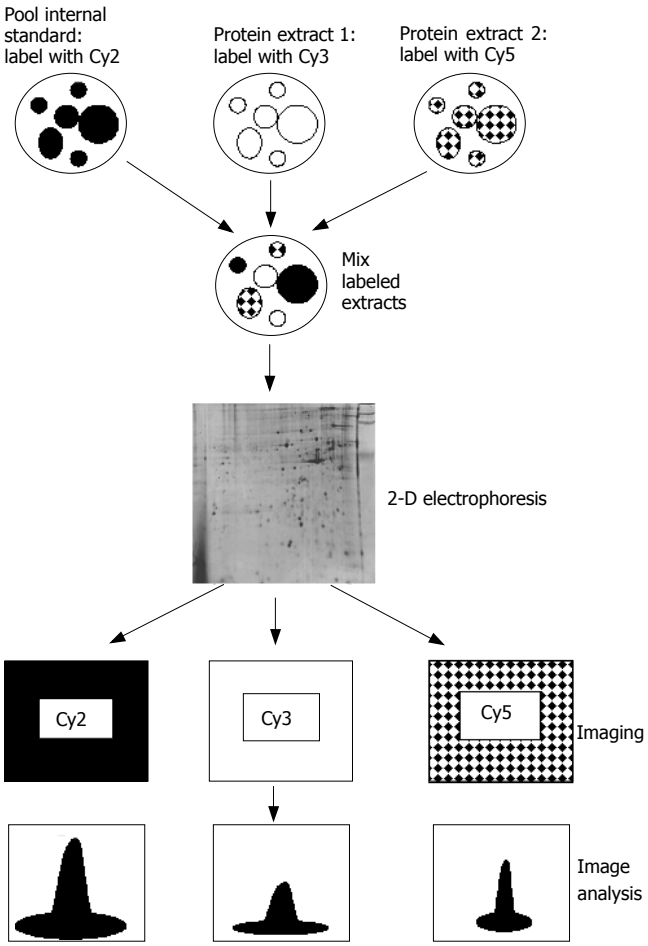

Figure 2 2D-DIGE techniques. Cy2, Cy3 and Cy5 are different fluorescent dyes.

of substrates have different affinities for different proteins, thus it is possible to increase protein representation when combining various arrays. The combination of these arrays with up-front prefractionation chromatography (e.g., anion exchange) permits the detection of up to 2000 protein species from serum[25,26]. The resulting spectral masses are analyzed using univariate and multivariate statistical instruments to provide a single marker or multimarker pattern that can classify clinical samples. Discriminator protein pinnacles are then purified and submitted to the MSbased identification process (Figure 4).

The SELDI technique was developed to profile clinical biological fluids, notably serum and/or plasma, and became important when numerous studies showed its potential in identifying unique biomarkers or complex patterns with diagnostic value, allowing its use for screening and early diagnosis in various cancers[27,28]. One major criticism of the technique relies on the overall lack of sensitivity and capability to detect tumor-specific protein traces within a large amount of nonspecific protein species[29]. However, even though still controversial in its reproducibility and ability to detect actual specific tumor signatures, SELDI has several advantages, such as easy of use, high throughput, and relatively reasonable cost, all making it a very attractive technique for working with large clinical sample.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), is a

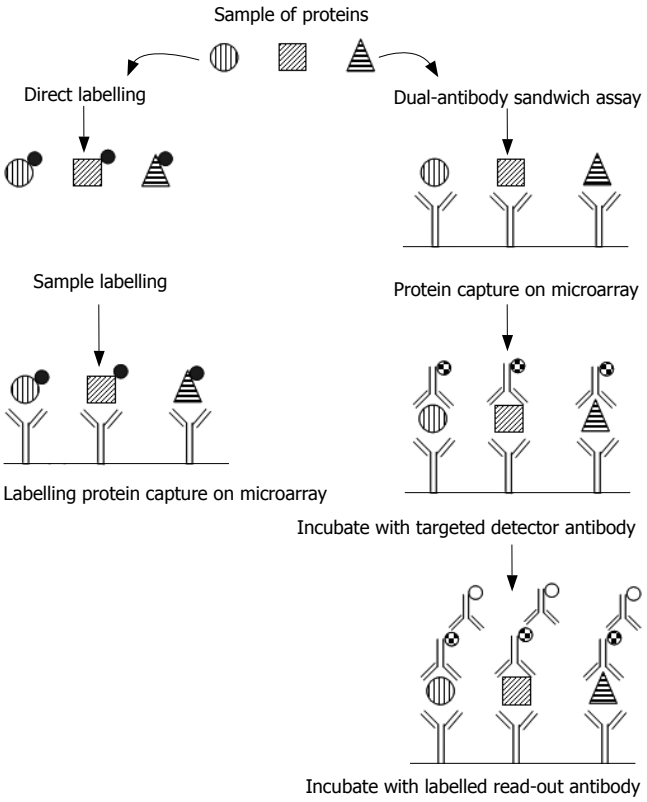

Figure 3 Representation of the two antibody microarray experimental formats. Direct labelling: single-capture antibody experiments; all proteins in a sample are labelled (black circles) thereby providing a means for detecting bound proteins following incubation on an antibody microarray. Dual-antibody (capture and read-out antibody) sandwich immunoassays: proteins captured on an antibody microarray are detected by a cocktail of tagged detection antibodies, which are matched to the spotted antibodies. The detector antibody tag is then measured by binding of a labelled (empty circles) read-out antibody.

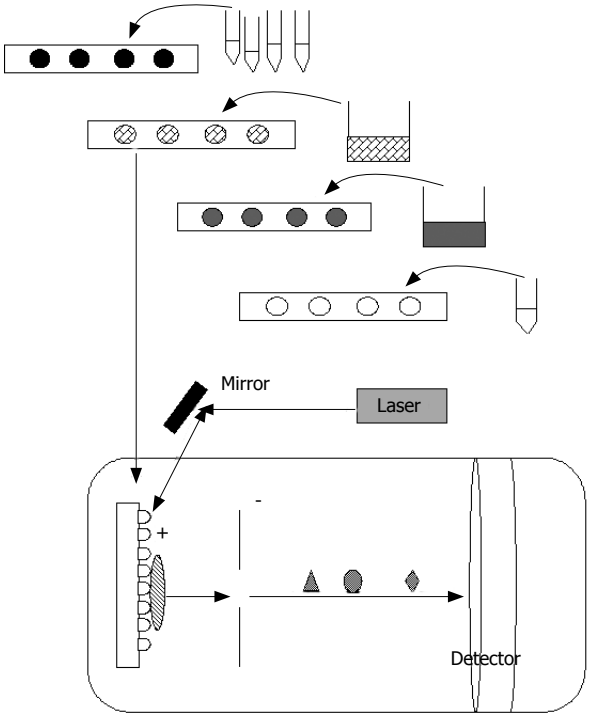

Figure 4 Principles of SELDI-TOF MS. The application of sample from to an eight-spot array with hydrophilic, hydrophobic, cationic, anionic or immobilized-metal affinity capture chromatography surface (black colour). The addition of an appropriate binding buffer (purple colour). On-chip sample purification using one or more wash buffers (grey colour). The application of energy-absorbing matrix for the absorption of laser energy (empty colour). Laser irradiation desorbs bound proteins and positively ionizes them. Owing to the electric field, they migrate in the mass analyser: (small diamond) and multiply charged proteins (oval) faster than large and single-charged ones (triangle). Thus, the proteins are separated. Time of flight (t) is proportional to protein mass per charge.

technique to analyze peptides and proteins in relatively complex samples. It has even been used for the direct analysis of tissue specimens[30]. In MALDI-TOF MS, a small quantity of specimen containing peptides and protein is dried on a target plate together with a light-absorbing matrix molecule.

Two technical advancements have improved resolution of MALDI-TOF MS to its current state. First, use of an electronic mirror (reflectron) to reflect ions substantially increases resolution, and second, delayed extraction introduced after sample vaporization and earlier than the electric potential is applied. Shorter times are optimal for small molecules, and longer times for large molecules. The standard detector for MALDI-TOF MS is a microchannel plate, which acts as an electron multiplier for ions reaching the detector. Detector replys relate to the number of ions reaching the detector and ion velocities.

MALDI-TOF MS permits a rapid determination of molecular masses and the heterogeneity of small amounts of peptides and proteins. Usually, intact molecular ions are formed and determination of polypeptide mass.

LC-MS and LC-MS-MS in comparative proteomic

Capillary-scale HPLC-MS/MS (LC-MS) is rapidly emerging as a method of choice for large scale proteomic analysis[31]. LC-MS systems can be used to identify and track the relative abundance of thousands of molecules[32]. For standard bottom-up profiling experiments, the molecules in question are peptides derived by proteolysis of intact proteins. For very complex protein samples, such as blood, the peptide mixtures are resolved by chromatographic separation prior to injection into the mass spectrometer. This generates a more informative map, that consists of both the unique elution of individual peptides. Distinct peptides of interest are induced by collision fragmentation followed by database matching for the purpose of sequence identification, while the recorded pattern of precursor ion intensities can be used to infer the relative quantities of the various proteins between samples[33].

LC-MS systems consists of different instruments to separate peptide mixtures based on physicochemical properties, separate ions on the basis of m/z ratios and registers the relative abundance of ions at discrete m/z.

In LC-MS-MS technique, precursor ions are recorded in full-scan mode, followed by selective ion isolation and fragmentation for sequence identification[33] (Figure 5).

Isotope-coded affinity tags (ICAT and iTRAQ)

This is the prototypical and the most popular method for quantitative proteome analysis based on stable isotope affinity tagging and MS[34].

The ICAT reagent is a sulphydryl-directed alkylating agent composed of iodoacetate attached to biotin through

a short oligomeric coupling arm (d0). The exchange of 8 deuterium atoms for hydrogen atoms in the coupling arm produces a heavy isotope version of the reagent (d8). Thus the reagent comprises of a cysteine reactive group, a linker containing the heavy or light isotopes (d8/d0) and a biotin affinity tag. This method involves *in vitro* derivatization of cysteine residues in protein with d0 or d8 followed by enzymatic digestion of the combined sample. All the cysteine residues thus tagged with biotin are selectively separated by avidin column and the cysteine-containing peptides are further separated followed by MS analysis[35].

The iTRAQ technique capable of multiplexing samples is primarily based on the ICAT technique and compared in detail. The iTRAQ technique uses four isobaric reagents allowing the multiplexing of four different simples in a single LC-MS-MS experiment. The multiplexing capability of iTRAQ allows a control sample to be compared with different points in time of a disease state, as well as with respect to different drug treatments. One of the major advantages of this technique is its ability to label multiple peptides per protein, which increases the confidence of identification and quantitation[16].

There are numerous differences (advantages and disadvantages) between the select proteomic technologies for protein profiling (Table 1).

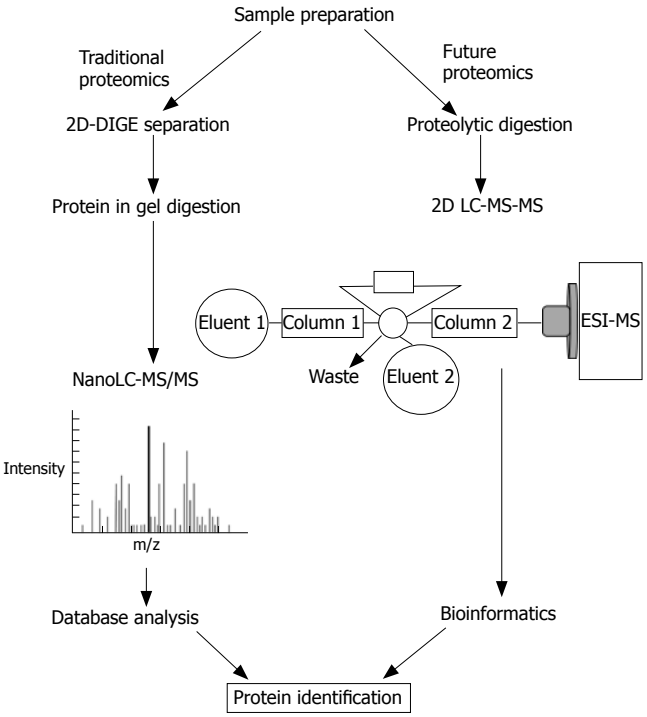

Figure 5 Different strategies for proteomic studies.

High-resolution hybrid quadrupole TOF

One of the first major advances used in any developing area of research was a high-resolution hybrid quadrupole TOF (QqTOF) MS fitted with a SELDI ion source to acquire proteomic patterns from serum. A recent study was designed to determine whether there is any diagnostic advantage provided by acquiring the proteomic patterns of serum samples using a high-resolution, high mass accuracy MS instrument. Results were analyzed on the exact same ProteinChip surface, thus eliminating all experimental variability apart from the use of two different instruments. Different combinations of bioinformatic heuristic parameters were used to generate different diagnostic models using the data acquired from the two distinct mass spectrometers[35]. These parameters included the similarity space for cluster classification, and the learning rate in training of the genetic algorithm. The diagnostic models generated from mass spectra acquired using the higher-resolution Qq-TOF MS were statistically superior[36].

Proteomic analysis software

The result of the analysis of a complex proteomic mixture by SELDI-TOF-MS is a low resolution profile of the protein or peptide species that were subsequently ionized from ProteinChip surface. It has been the development and combination of sophisticated bioinformatic algorithms for the analysis of SELDI-TOF-MS data. The intention of this bioinformatic analysis has led to the potential application of this technology as a major advancement in the diagnosis of cancer and other diseases. There are several different types of bioinformatic algorithms, such as single classification trees, neural nets, genetic algorithms, and random forest algorithms, which have been applied to enable SELDI-TOF-MS data to be investigated as a diagnostic technology. Although they function in different protocols, these algorithms share a common goal: to construct a classifier and discover peak intensities most likely to be responsible for segregating classes of samples[37]. Since its inception, SELDI-TOF-MS has been used to develop diagnostic platforms for several different cancers.

PROTEOMIC ANALYSIS IN COLORECTAL CANCER

During the past decade, genomic analyses have been introduced into cancer studies with variable success. It has become recognized that genomic techniques are insufficient to study the complex pathways of carcinogenesis; this has led to the application of proteomic techniques, which allow for the reliable analysis of complex mixtures of proteins[38].

Colorectal cancer is the third most common cancer in the world. It is well known that the adenomatous polyposis coli (APC) gene is mutated in patients with familial adenomatous polyposis (FAP) and sporadic colorectal cancer, and that mutations initiate colorectal carcinogenesis. It is now suggested that many colorectal cancers arise from preexisting adenomas. Following several steps of mutation of oncogenes and tumor suppressor genes, adenomas develop to colorectal cancers[7].

Many groups have reported the proteomic analyses of colorectal cancers. Dundas *et al*[39] found that mortalin, also known as mitochondrial HSP70, is involved in cell cycle regulation with important roles in cellular senescence and immortalization pathways and was over-expressed in colorectal adenocarcinomas and correlated with poor survival. Lane *et al*[40] identified over-expressed multiple cytochrome P450 enzymes in human colorectal cancer tissues and metastases. Cytochrome P450 proteins (CYPs)

Table 1 Advantages and disadvantages of proteomic technologies for protein profiling

Technique	Methods	Advantages	Disadvantages
2D	Separation on a gel of the protein content of a sample in two dimensions according to mass and charge; gels are stained and spot intensities in samples are compared among different gels	High separation (thousands of proteins per gel)	Low throughput laborious (one samples per gel); poor resolution for extreme masses and extremely acidic or basic proteins; no direct protein identification; large amount of starting material compared with other techniques
2D-DIGE	Measuring three samples per gel; each of them is labelled with a different fluorescent dye, and the intensities of each gel spot for each sample are measured at a wavelength specific for the label	Direct comparison of samples on one gel: better reproductibility	Low throughput (three samples per gel)
Protein microarrays	Binding of a targeted protein in one sample to spotted probes on a 'forward' microarray; conversely, binding of specific probes to a targeted protein in spotted samples on a 'reverse' microarray; detection of bound proteins by direct labelling or by labelled secondary antibodies	High throughput in terms of number of probes per (forward) array or number of samples per (reverse) array; biomarker identity or class readily known	Synthesis of many different probes necessary; identity or class of targeted proteins must be known; limited to detection of proteins targeted by the probes
SELDI-TOF MS	Selected part of a protein mixture is bound to a specific chromatographic surface and the rest washed away	High throughput; direct application of whole sample (fast on-chip sample cleanup); small amount of starting material	Unsuitable for high molecular weight proteins; limited to detection of bound proteins; lower resolution and mass accuracy than MALDI-TOF
MALDI-TOF MS	Application of a protein mixture onto a gold plate; desorption of proteins from the plate by laser energy and measurement of the protein masses; comparison of peak intensities between multiple samples	High throughput	Need for sample fractionation of complex samples; more starting material needed for sample fractionation; unsuitable for high molecular weight proteins
LC-MS-MS	Separation of a mixture of peptides (resulting from protein digestion with trypsin) by one-, two-or three-dimensional LC and measurement of peptide masses by MS-MS	Direct identification of several hundred proteins per sample by MS-MS of peptides	Low throughput; time consuming; detection by MS–MS often not comprehensive, tus complicating comparison of different samples
ICAT	Chemical tagging of proteins on cysteine residues with a heavy or light stable isotopic; after labelling samples are mixed, proteins are digested with trypsin, and labelled peptides isolated by affinity chromatography; both samples are analysed concomitantly by LC-MS-MS	Direct identification of biomarkers by MS-MS of peptides; relative quantitation; less sample complexity than with iTRAQe; MS-MS of only differentially expressed proteins	Low throughput; tagging of only cysteine-containing peptides
iTRAQ	Chemical tagging of proteins on their amine groups with stable isotopic labels of identical mass ('isobaric'); four different labels are available for four different samples; after labelling, samples are mixed, proteins digested with trypsin and analysed concomitantly by LC-MS-MS	Direct identification of biomarkers by MS-MS of peptides; owing to isobaric labels, selection for MS-MS of the same peptide in all four samples in the same single MS run	Low throughput (four samples per run); for generating signature ion, MS-MS of all peptides in a sample is necessary; high sample complexity and limited resolution of LC (even three dimensional), confounding by co-eluting isobaric peptides

in the liver are known to be of major importance to the fate of anticancer agents; however, their expression and role in tumours has received little attention. CYP-mediated metabolism is generally viewed as a route to drug detoxification and increased elimination, although CYP activation of certain anticancer drugs. The presence of metabolically active CYPs in a colon metastastic deposit is likely to be important in determining the metabolic fate of chemotherapeutic agents and hence the outcome of treatment. Stulik *et al* performed proteomic differential display between the matched sets of macroscopically normal colon mucosa and colorectal cancer tissues. They report that the expression of HSP70, S100A9, S100A8, S100A11 and S100A6 was up-regulated in colorectal cancer tissues compared to normal colon mucosa, and the levels of liver fatty acid-binding protein, actin-binding protein/smooth muscle protein 22-a and cyclooxygenase 2 were down-regulated in transformed colon mucosa[41]. The S100A6 protein was the first S-100 protein specifically identified as being related to the state of cellular proliferation. The possible correlation between increased expression of some members of the S100 protein

Table 2 Proteomic analysis in human colorectal cancer tissues

Up-regulated		Down-regulated
Annexin Ⅳ		NCF2
MTA-1		PMM2
SSX5 protein		Serpin 1
Dynein heavy chain		CNRC
Cytochrome P450		Annexin Ⅴ
CPT1		APC
Keratin 10		VAV3 protein
Keratin 8		RSP 4
Keratin 19		SPARC like protein 1
Vimentin		PDI
β-actin		GN6ST
REL1		Cathepsin D
HSP60		Calreticulin
Mortalin	Cathepsin fragment	SM31
		PDA6
	Proteasome subunit a type 6	ApoA1 precursor
Cytochrome P450 enzymes		ATP synthase b chain
(in cancer tissues and metastatic tissues)	Triosephosphate isomerase 14-3-3 proteins	Albumin
		Liver fatty acid-binding protein
HSP70	GST-P	Actin-binding protein/smooth muscle protein 22-a
S100A9		
S100A8	P13693 translationally controlled tumor protein	Cyclooxygenase 2
S100A11		
S100A6		Puromycin-sensitive aminopeptidase
	Nucleoside diphosphate kinase A	
		NADH-ubiquinone oxidoreductase
Adenosyl homocysteinase	Calgranulin B; S100 A9	
Leukocyte elastase inhibitor, claude B		Succinate dehydrogenase subunit A
Macrophage capping protein		
Biliverdin reductase A		Aldehyde dehydrogenase, cytosolic, class Ⅰ
Annexin 1 fragment		
α-tubulin		
Elongation factor 1-d		Selenium-binding protein
Tropomyosin a1		Creatin kinase B chain
Tropomyosin a4 chain		Placental thrombin inhibitor
Actin fragment		Vimentin
Annexin 5		Desmin
Microtuble-associated protein RP/EB		Tubulin b 5 chain
Pyridoxal kinase		Carbonic anhydrase Ⅰ
Annexin 3		Myosin regulatory light chain 2
Annexin 4		

family and colon carcinogenesis is also supported by the finding that documents the participation of the S100A4 protein in the progression and metastasis of colorectal carcinogenesis. Alfonso *et al*[42] reported the up-regulation of annexin Ⅳ, MTA-1 and others in colorectal cancer tissues, and the down-regulation of NCF2, PMM2 and others. Several functional groups of proteins were affected, including regulators of transcription, structural proteins, and those involved in protein synthesis and folding. The MTA-1 gene encodes a protein that was identified in metastatic cells, specifically, mammary adenocarcinoma cell lines. Expression of the MTA-1 gene has been associated with the progression of several carcinomas in colon, lung, prostate, and liver. A annexin Ⅳ is a calcium-binding protein and I involved in cellular communication and signal transduction, for this reason it was up-regulated in colorectal cancer. Friedman *et al*[43] identified adenosyl homocysteinase, leukocyte elastase inhibitor and others as up-regulated proteins, and puromycin-sensitive aminopeptidase, NADH-ubiquinone oxidoreductase and others as down-regulated proteins in colorectal cancer tissues.

Minowa *et al*[44] identified truncated β-tubulins as a protein specific to polyp samples from APC gene-mutant mice by proteomic analysis of the small intestine and colon epithelia. The adenomatous polyposis coli gene (APC) is mutated in patients with familial adenomatous popyposis (FAC) and sporadic colon cancer, and these mutations initiate colon carcinogenesis. Simpson *et al*[45] performed membrane proteomic analysis of the human colon carcinoma cell line LIM 1215 to search for novel tumor marker proteins expressed during various stages of cancer progression, although the data are not shown.

Given the continual rise in the number of potential biomarkers of CRC, future studies will increasingly employ genomic and proteomic technologies, which enable the measurement and analysis of numerous potential biomarkers simultaneously. These techniques are able to produce gene or protein 'profiles' associated with clinical outcome, the analysis of which may then yield novel biomarkers with prognostic and/or therapeutic potential[46] (Table 2).

At this moment, biomarkers whose sensitivity and

specificity are better than bloody stool examination have not yet been found. Since the bloody stool test is easier than examination using cancer specimens and easier to handle than sera, from a clinical aspect, the bloody stool examination is better than biomarkers[34].

In another recent study, the detection of upregulated α-defensins 1, 2 and 3 in colorectal cancer tissue were reported in two independent, but similar analyzes. In both studies, SELDI-TOF MS results in tissue correlated with serum levels that were determined using ELISA or SELDI-TOF MS. This provides an interesting approach for finding new serum markers because biomarkers identified first in tissue could prove to be more specific. Unfortunately, α-defensin levels are also increased in serum during, for example, infection[47]. α-defensin and β-defensin are major components of the epithelial mammalian innate immune system. Defensins are small cationic peptides with high activity against a variety of microbials, encoded by genes and some are regulated in response to challenge with bacterial antigens. Gastrointestinal α-defensins (HD5 and HD6) are almost exclusively expressed in and secreted from Paneth cells of the small intestine, while β-defensins (hBD-1, hBD-2, hBD-3) are secreted by virtually all gastrointestinal epithelial cells to a varying extent.

CONCLUSIONS AND FUTURE PERSPECTIVES

Rapidly developing techniques that considerably enhanced information gained from proteomes integrate proteomics with other disciplines such as cell biology, biochemistry, molecular genetics, and chemistry. This consolidation certainly demonstrates incredible power and possibilities of proteomics for further applications. It is necessary to cross the barriers of limited resolution, mass range, detection level, and other reasons for protein underrepresentation in analyzed proteomes. Once achieved, the door that allows complete identification of specific protein markers will open and the comprehension of complex networks of protein/peptide interactions involved in cancer will begin to be elucidated[6]. While the application of computational and statistical methods to proteomic profiling is relatively new, it is rapidly gaining interest. Hence, it is worthwhile suggesting fruitful avenues for moving forward. It was suggested above that simultaneous LC-MS data alignment and normalization may be beneficial for comparative profiling.

Proteomic technologies are now in place to examine simultaneously and comprehensively many protein expression differences that result from disease and treatment, with the ultimate payoff being the use of specific protein profiles for the early diagnosis of patients and for patient-tailored therapies[47].

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Immunotherapy and immunoescape in colorectal cancer

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Abstract

Immunotherapy encompasses a variety of interventions and techniques with the common goal of eliciting tumor cell destructive immune responses. Colorectal carcinoma often presents as metastatic disease that impedes curative surgery. Novel strategies such as active immunization with dendritic cells (DCs), gene transfer of cytokines into tumor cells or administration of immunostimulatory monoclonal antibodies (such as anti-CD137 or anti-CTLA-4) have been assessed in preclinical studies and are at an early clinical development stage. Importantly, there is accumulating evidence that chemotherapy and immunotherapy can be combined in the treatment of some cases with colorectal cancer, with synergistic potentiation as a result of antigens cross-presented by dendritic cells and/or elimination of competitor or suppressive T lymphocyte populations (regulatory T-cells). However, genetic and epigenetic unstable carcinoma cells frequently evolve mechanisms of immunoevasion that are the result of either loss of antigen presentation, or an active expression of immunosuppressive substances. Some of these actively immunosuppressive mechanisms are inducible by cytokines that signify the arrival of an effector immune response. For example, induction of 2, 3 indoleamine dioxygenase (IDO) by IFNγ in colorectal carcinoma cells. Combinational and balanced strategies fostering antigen presentation, T-cell costimulation and interference with immune regulatory mechanisms will probably take the stage in translational research in the treatment of colorectal carcinoma.

Key words: Colorectal carcinoma; Immunotherapy; Gene therapy; Interleukin-12; Dendritic cells; CD137; Indoleamine 2, 3 dioxygenase

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INTRODUCTION

Conventional therapy for cancer is based on surgical resection, chemotherapy with drugs with selective toxic effects against dividing cancer cells, and localized gamma irradiation. Biological therapy has only recently been introduced[1]. This includes the use of agents that interfere with growth factors for malignant cells, and block tumor neovascularization[2]. Among the monoclonal antibodies (mAbs) that have been approved for cancer treatment, most operate *via* indirect mechanisms, and only a minority target natural or artificial mechanisms of cell destruction.

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related deaths worldwide[3]. Unfortunately, more than 20% of patients with CRC have metastatic disease at the time of diagnosis (http://www.seer.cancer.gov). Although the most common indication for liver resection in developed countries is metastatic CRC, surgery can only be performed in 20% patients, with the 5-year survival rate of 25%-40% despite adjuvant chemotherapy[4]. Regardless of this depressing scenario, a better understanding of tumor biology, combined with advances in molecular and cell biology, have opened up novel avenues of treating advanced CRC using immunotherapeutic strategies.

Tumor escape: Perverted local and systemic immune regulation by tumors

The cellular immune system has been endowed with powerful and at the same time toxic mechanisms designed to induce inflammation and cell destruction, which should be kept under tight control and guided precisely to the target tissues. Cytotoxic mechanisms are designed to recognize and destroy cells that are infected with viruses or other intracellular pathogens, whereas inflammation

is a vascular and leukocyte mediated local response that selectively directs the cellular and macromolecular elements of the innate and adaptive immune systems to the infected site. If properly aimed and enhanced, both immune functions can be therapeutically exploited to control and even eradicate malignant lesions[5]. Genetic and epigenetic changes involved in carcinogenesis generate antigens that are recognized by T lymphocytes in analogous fashion to microbial antigens[6]. Unfortunately, tumor cells in spite of being antigenic are very poorly immunogenic by themselves. Therefore, advanced cancer disease can impede any effort to induce antitumor immunity.

Genetically unstable cells can undergo genetic or epigenetic changes in order to escape a tumoricidal immune response in a "survival of the fittest" type of selection. The escape mechanisms may result from loss of antigen or antigen presentation as well as from active biosynthesis of immunosuppressive molecules[7,8]. These factors include TGF-β, VEGF, IL-8 and IL-10 which are known to cause significant inhibition of both innate and adaptive mechanisms of tumor immunity. Recent evidence points to activation of the transcription factor *Stat3* as a master switch in the control of various immunoevasive substances in tumor cells[9]. Moreover, intrinsic *Stat3* signaling in hemopoietic cells hindered their performance in tumor immunity including dysfunction of NK cells, granulocytes, and conventional DCs which become tolerogenic. Infiltration of tumors by effector T cells seems largely an inefficient process that may be related to poor expression of chemokines and vascular adhesion molecules in the malignant lesions[10]. Besides, the myeloid and lymphoid cells present in tumor stroma appear to be related more to the mechanisms of inhibition than to the activation of tumor immunity.

Indoleamine 2, 3 dioxygenase (IDO) catalyses the degradation of the essential amino acid tryptophan and synthesizes immunosuppressive metabolites[11]. Local up-regulation of the expression and activity of IDO in tumors and the draining lymph nodes can suppress T cell activation and is thought to facilitate the escape of tumor cells from the immune system[12]. Indeed, this enzyme depletes tryptophan and produces kynurenines locally in such a way that both mechanisms impair the function of T cells[13]. IFNs are the key factors upregulating IDO, thus generating a clever mechanism that becomes operational when tumors sense an active immune response in their neighborhood. There is recent evidence indicating that upregulation of IDO by colorectal cancer cells provides an immunosuppressive microenvironment created by tumors to promote cancer growth and spread[14]. We have observed in *in vitro* studies that the addition of IFN-γ to CT26 murine colorectal carcinoma cells induces IDO mRNA expression as well as IDO enzymatic activity, detected as kynurenine production (Figure 1).

Co-signaling molecules are cell-surface glycoproteins that can direct, modulate and fine tune T-cell receptor (TCR) signals[15]. The functional outcome of T cell activity upon its binding to a ligand on an adjacent cell membrane classifies co-signaling molecules as co-stimulators and co-inhibitors. Tumors can express co-inhibitory B7 family

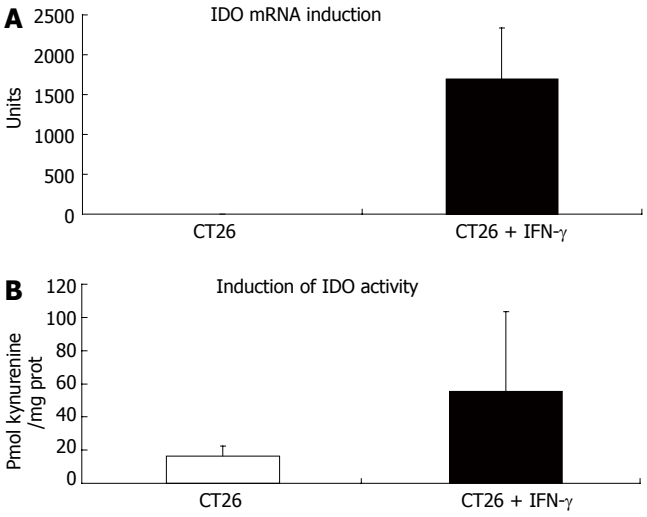

Figure 1 IFN-γ induces IDO mRNA and enzymatic activity in colon cancer cells. **A**: IDO mRNA was induced after 48 h stimulation with 1000 IU/mL of IFN-γ, as assessed by real time-PCR; **B**: In the same culture conditions, IDO activity was measured in CT26 cellular extracts as previously described by Takikawa *et al*[83].

members, such as B7-H1, B7-H4, and B7-1 (CD80) at a low density, which downregulates T cell activation and/or cytolytic activity[16,17]. Tumors can also induce B7-H1 and B7-H4 expression on tumor-associated macrophages (TAM)[18]. Myeloid suppressor cells can further inhibit anti tumor T cells *via* the production of nitric oxide by the enzyme arginase[19].

Regulatory T cells (T-reg) are important inhibitors of anti tumor immunity[20]. T-reg, characterized by the FoxP3 transcription factor, up-regulate a number of cell membrane molecules, including LAG-3, CTLA-4, GITR, and neuropilin. T-reg can inhibit effector T cell activation and function *via* T-T inhibition or inhibition of antigen presenting cells. There is experimental evidence to support a grim scenario in which T cells in tumor tissue or draining lymph nodes can be perverted into regulatory T cells[21]. Local production of TGF-β may be a key factor in transforming effector T cells locally into suppressive T-reg. Convincing data concerning the role of $CD4^+$ $CD25^+$ regulatory T cells in human cancer comes from the work of Curiel *et al*[22], who showed that the presence of such T-reg in advanced ovarian cancer correlated with reduced survival. Considering the role of T-regs as inhibitors of anti tumor immunity, it has been observed in murine models and in patients that prior host immunosuppression with chemotherapeutic agents (such as cyclophosphamide) can increase the efficacy of adoptive cell therapy as well as other kinds of immunotherapy[23]. The reason for this immunomodulatory effects is based, at least partially, on the elimination of $CD4^+$ $CD25^+$ T cells and the engraftment of specific cytotoxic T lymphocytes[24].

Experimental evidence with TCR transgenic mice clearly shows that tumor-reactive T cells can be tolerized to the point where there is no response to the surrogate tumor antigen. Tolerance results from presentation in the context of a DC that is not expressing high levels of costimulatory molecules and does not secrete cytokines such as IL-12, IL-15 and IFNs.

Chronic exposure to high levels of antigen drives T lymphocytes to a state of non-responsiveness termed "exhaustion". This phenomenon may play a role in impaired CD8 T-cell activity in response to persistent tumor antigens. In a way, the phenomenon of CD8 T-cell exhaustion is actually encouraging from the perspective of immunotherapy, since tumor-specific CD8 T-cells may be present and partially primed in a tumor-bearing host. The B7H1 and PD1 ligand receptor pair is a clear candidate to mediate and sustain exhaustion and offers an opportunity for therapeutic intervention.

In many cases however, a responsive TCR repertoire and tumor antigens coexist without signs of immunization or tolerization. Such a situation is termed immunological ignorance or indifference[25]. Ignorance can conceivably take place in two different ways. First, the quantity of antigen presented to the lymphoid tissue may be too small to induce immunity or tolerance. That would be ignorance/indifference at the priming phase of the immune response[26]. Second, studies in mice show that an expanded effector cell population respects tissues that are not inflamed[27,28]. This can be termed ignorance at the peripheral level that can occur in peripheral solid tumors[28,29].

The possibility of overcoming immunoescape

Immunotherapy, which is an intervention designed to increase anti-cancer immunity, remains an experimental discipline[30]. However several approaches including inducing and redirecting immunity to either the malignant cells or to critical components of the tumor stroma, such as the vasculature or the connective tissue, have been shown to profoundly impact disease progression in mouse models of cancer[31,32].

Therapeutic vaccination has been attempted in several ways. The immunogenic source can be autologous or allogenic malignant cells that are modified to increase their immunogenicity[33]. *Ex-vivo* or *in vivo* gene transfer of cytokines and other immune-potentiating molecules is a promising strategy. Alternatively, many experimental protocols rely on *in vitro* culture/differentiation of DCs manipulated in such a way that they artificially present tumor antigens[34]. However, the promising results in mouse models have not been replicated in clinical trials. In spite of this drawback there is ample biological evidence in humans that there is an increase in the numbers and activity of lymphocytes against the vaccinating antigen, although such increases fail to reach by 1-2 logs the levels of T cell immunity observed in viral infections.

Adoptive T cell therapy with activated T lymphocytes reaches higher levels of circulating antitumor T cells[35]. These techniques are based on *ex-vivo* reactivation and expansion of cloned or polyclonal cultures of tumor reactive T cells. After culture, T cells are reinfused into the patient along with IL-2. Three important concepts have gained experimental support: (1) polyclonal cultures that recognize several antigen specificities improves the outcome, and the development of tumor-escape antigen loss variants are less likely to occur, (2) co-infusion of both CD4 and CD8 tumor reactive T cells improves antitumor activity, and (3) treatment with lymphodepleting chemotherapy before reinfusion increases the duration and *in vivo* re-expansion of the infused T cells. This is due to both depletion of regulatory T cells and decrease in the competition for T cell homeostatic survival factors such as IL-15 and IL-7. Adoptive T cell therapy probably will benefit much more from the availability of clinical grade IL-15, which can condition the infused cells and sustain their function on administration to the patient.

The sense that chemotherapy and immunotherapy are incompatible is a fading paradigm in tumor immunotherapy. It used to be reasoned that if T cell responses require cell expansion, active or adoptive immunotherapy could not be used in combination with chemotherapy drugs that are selectively toxic for dividing cells. Several lines of experimental evidence suggest otherwise. In fact, there are a number of mechanisms that define additive and synergistic effects: (1) tumor cell destruction makes tumor antigens available for cross presentation by DCs, (2) there is decrease in regulatory T cells, and (3) there is reduced competition for T-cell homeostatic growth factors during/after active immunization. Local destruction of tumors followed by injection of proinflammatory substances holds much promise according to preclinical data and probably represents the simplest method of converting tumors into tumor vaccine.

Immunostimulatory monoclonal antibodies for the treatment of colorectal carcinoma

Immunostimulatory mAbs directed to immune receptors have emerged as a new and promising strategy to fight cancer[36]. In general, mAbs can be designed to bind molecules on the surface of lymphocytes or antigen presenting cells to provide activating signals (e.g., CD28, CD137, CD40 and OX40)[36]. On the other hand, mAbs can also be used to block the action of surface receptors that normally downregulate immune responses (CTLA-4 and PD-1/B7-H1). In combined regimes of immunotherapy, these mAbs are expected to improve therapeutic immunizations against tumors as observed in preclinical studies.

Anti-4-1BB (agonistic anti-CD137) is one of the most interesting mAbs tested as anti-cancer molecules in preclinical studies[36]. 4-1BB is a member of the tumor necrosis factor/nerve growth factor family of receptors and has a natural ligand (4-1BBL) that is expressed on activated T lymphocytes as well as on NK cells and dendritic cells[37]. This mAb, which acts against CD137, has the ability to stimulate potent antitumor responses[38] and, paradoxically, ameliorates autoimmune manifestations in mice[36]. On the other hand, therapy with mAbs against CTLA-4, which block the inhibitory action of CTLA-4 on T-cells, is capable of inducing antitumor responses in mice as well as in humans but is accompanied with adverse events in the form of autoimmune reactions[39].

Kocak *et al*[40] took advantage of both the mAbs and showed that the combination of CTLA-4 and 4-1BB acts synergistically in the eradication of MC38 colorectal carcinoma after stimulation of a potent antitumor immune response. It was observed that this antitumoral effect

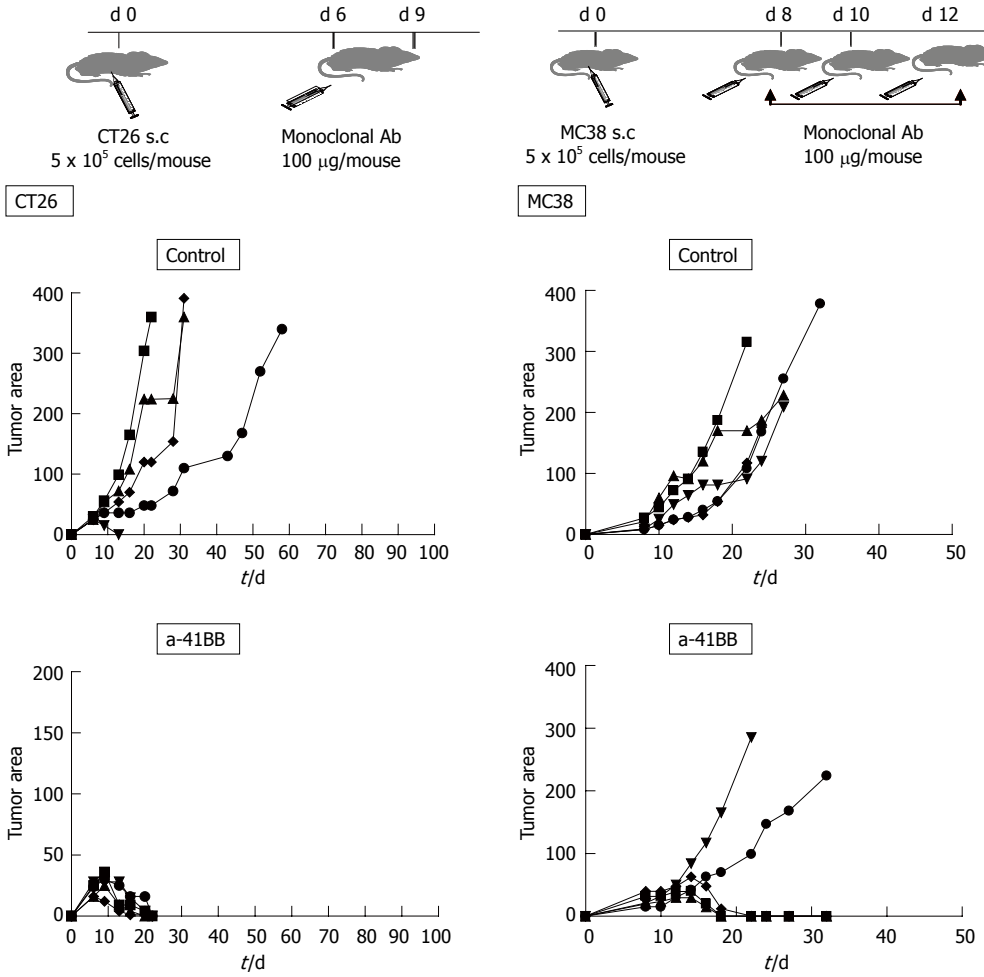

Figure 2 Systemic treatment with agonist anti-CD137 monoclonal antibodies eradicates transplanted murine colon cancers. Mice subcutaneously grafted with 5 x 10^5 CT26 or MC38 cells were treated with anti-CD137 (2A) mAb or polyclonal rat IgG as a control. Sequential follow up of tumor size (mean diameter) is depicted for individual mice.

is critically dependant on the presence of $CD8^+$ T-cells induced after treatment[40]. However, we did not observe such a synergy in the same experimental model (A Arina *et al*, unpublished observations).

In our studies in mice, we used the MC38- and CT26-derived tumor model (colorectal carcinoma cell lines) to explore the antitumor effect of repeated systemic injections of agonistic anti-CD137 (anti-4-1BB) mAbs. As a result of the amplification properties of anti-CD137 antibodies on CTL immune response, this treatment was able to induce tumor eradication in 3 out of 5 mice bearing CT-26 tumors and in 3 out of 5 animals with MC38 nodules (Figure 2).

CD137 stimulation can be achieved not only by direct administration of mAbs in monotherapy, but also in the context of different combinations usually including immunostimulatory cytokines. For example, simultaneous gene transfer of local-membrane bound 4-1BB ligand and IL-12 results in successful eradication of advanced colorectal liver metastasis induced in mice[41]. In a similar line of work, Martinet *et al*[41] demonstrated that the combination of 4-1BB costimulation using an adenovirus expressing membrane-bound 4-1BB-L with another adenovirus expressing IL-12 genes induced a potent antitumor response in mice with colorectal carcinoma. Systemic administration of soluble Ig-4-1BB ligand gave rise to a stronger T-cell immune response compared to local gene transfer[42]. It appears that anti-4-1BB can upregulate a formerly weak immune response, but it fails to initiate an immune response if it was nonexistent initially[43].

Systemic treatment with anti CTLA-4 mAb increased the number of CTLs and caused complete tumor regression in established colorectal carcinoma in mice[44]. Another attractive immunostimulatory combination was recently examined by Tirapu *et al*. These workers searched for strategies to enhance the efficacy previously achieved by intratumoral injection of DCs engineered to secrete IL-12 in a mouse model of colorectal carcinoma (using MC38 cell line). They were able to induce a systemic immune response (measured by IFN-γ ELISPOT assay) that eradicated large and metastatic tumor lesions using a combination of systemic anti-CD137 mAb and IL-12 producing semiallogeneic DCs injected intratumorally[45]. This study offers a promising technique of enhancing the efficacy of DC-based strategies currently been tested in clinical studies[46].

GENE TRANSFER OF IMMUNOSTIMULATORY MOLECULES AND GENETIC VACCINATION

Several cytokines (e.g., IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IFN-γ, TNF-α and GM-CSF) demonstrate an ability to increase anti-tumor immunity when expressed by cancer

Table 1 Gene transfer of immunostimulatory molecules and genetic vaccination

Cytokine	Vector	Clinical application	Mechanism	Ref.
IL-2 + IL-12	Ad	No	CTLs	10
IL-10	Retrovirus	No	$CD8^+$	54
TNF-alpha	Ad	Yes	Antiangiogenic, bystander effect	75
HLA-B7/b2 microglobulin	DNA	Yes	CTLs	76
IL-12	Ad	Yes	NK, $CD4^+$, $CD8^+$	46
IL-12 + IL-10	Retrovirus	No	$CD8^+$, $CD4^+$, NK, Macrophages, Neutrophils	55
IL-2	Ad, retrovirus	Yes	CTLs	59
CCL21/LIGTH	Ad	No	DC, $CD8^+$, Macrophages	61

Ad: Adenovirus; DNA: Plasmid DNA; CTLs: Cytotoxic T lymphocytes; NK: Natural killers.

cells[47]. However, systemic administration of recombinant cytokines has limitations because of their short half-life, production difficulty and toxicity. Gene therapy appears to be a novel strategy that may help in delivering therapeutic genes locally, as well as the possibility of controlling transgene expression using specific and regulatable promoters[48] (Table 1).

Currently, we consider two principal approaches to the transfer of immunostimulating molecules inside tumors in order to facilitate immunity against colorectal cancer[47]: (1) *in vivo* injection of vectors expressing cytokines/costimulatory molecule genes into the tumor milieu (may be the most straightforward technique), and (2) tumor cells, DC and lymphocytes can be transduced *ex vivo* with vectors encoding cytokines/costimulatory molecules and re-administered into the host. One of the aims of these strategies is to induce high tumoral or peritumoral production of transferred cytokines, to promote localized regional inflammation (to stimulate innate anti-tumor response), and to induce systemic immunity capable of eliminating disseminated disease.

One of the most extensively studied cytokines in cancer treatment is interleukin-12 (IL-12), which has been shown to have significant antitumor activity against a wide panel of experimental malignancies. IL-12 promotes antitumor immunity because of its ability to activate cytotoxic T lymphocytes (CTLs), natural killer (NK cells) and Th1 response[49,50]. Moreover, IL-12 has antiangiogenic effect, dependent on Interferon gamma (IFN-γ) Inducible Protein 10 (IP10) that facilitates its anticancer effect through different mechanisms[51,52]. It is well known that systemic therapy with rIL-12 protein carries the risk of severe toxicity because of the stimulation of large quantities of IFN-γ, with the potential for individually heterogeneous susceptibility[53].

It has been observed that a combination of immunostimulatory genes may achieve superior therapeutic effects. Narvaiza *et al*[51] demonstrated that intratumoral administration of an adenovirus encoding IL-12 (AdIL-12) together with another adenovirus encoding the chemokine IP-10 (AdIP-10) results in marked antitumoral synergy leading to eradication of metastatic colorectal carcinomas. In this study, the authors used vectors in doses that were not effective when given separately. Moreover, this strategy allowed reduction in the dose of AdIL-12 without losing its anti-tumor efficacy and with less risk of IL-12-related toxicity[51]. The underlying principle of combining AdIL-12 and AdIP-10 is based on the prospect of attracting lymphocytes to tumors expressing IP-10 and to activate them by simultaneous infection of the tumor with AdIL-12.

It is well known that IL-12 has the ability to induce a Th1 type of immune response. By contrast, IL-10 is mainly expressed by Th2 cells and downregulates the production of IL-12 by antigen presenting cells, thus decreasing Th1 activity[51]. However, it has been observed that IL-10 enhances IL-2-induced proliferation and differentiation of $CD8^+$ T-cells[29]. Adris *et al*[54] showed that inoculation of mice with tumor cells expressing IL-10 inhibits the establishment of colorectal carcinoma cells and induces a T cell-mediated tumor suppression in the context of a systemic Th2 response. In an effort to treat colorectal carcinomas using both cytokines, Lopez *et al*[55] have shown that tumor cell vaccines producing both IL-10 and IL-12 act synergistically to eradicate established colorectal cancer (CT26 cell line) and, surprisingly, mammary carcinomas as well. The authors also observed that the antitumor effect of the combined immunotherapy was mainly dependent on $CD8^+$ cells.

In addition to IL-12, heat shock proteins (HSPs) also have the ability to stimulate antigen-presenting cells and induce a Th1-type response. HSP have been employed as an adjuvant to facilitate the induction of specific immunity. Moreover, HSPs have been evaluated in clinical studies as an adjuvant in combination with BCG (Bacille Calmette-Guerin) and HPV16E7 in patients with papillomavirus-related carcinoma[56]. Wu *et al*[57] demonstrated that vaccination of transgenic mice with HSP70-like protein (Hsp70L1) fused with a fragment of carcinoembryonic antigen (CEA576-669) induced the maturation of DCs, with a strong specific CD8 T cell response and *in vivo* antitumor activity in mice.

Systemic administration of recombinant IL-2 has been used in clinical practice in patients with metastatic renal carcinoma and malignant melanoma, although with low efficacy and high toxicity[58]. Among other functions, IL-2 is necessary for the survival of activated T cells and is employed in large doses in protocols were immune cells are adoptively transferred to cancer patients. Adenovirus containing mouse IL-2 cDNA can be injected into tumors, and in combination with a suicide gene (herpes simplex virus thymidine kinase vector) can be a powerful tool in the treatment of metastatic colon carcinoma of the liver[59].

One of the synergistic combinations include a chemokine plus a T-cell-activating cytokine designed to promote the attraction and activation of infiltrating immune cells (attraction theory). Macrophage inflammatory protein 3 (MIP-3) is a chemokine mainly secreted by activated macrophages, which attracts leukocytes to inflammatory foci with selectivity for tisular

DCs. The combination of two adenoviruses, one encoding MIP-3 (Ad MIP-3) and the other IL-12 genes (AdIL-12) given intratumorally in mice with colorectal carcinoma eradicates nearly 90% of subcutaneously implanted tumors[60]. Similarly, co-expression of the chemokine CCL21/secondary lymphoid tissue chemokine and a costimulatory molecule LIGHT in colon carcinoma cells (CT26) resulted in significantly reduced tumor growth in mice. A markedly increased infiltration of mature DCs and $CD8^+$ T cells was observed in the tumor mass, and the splenocytes showed a potent CTL activity against CT26 tumor and IFN-γ production. These results suggest that combined treatment with CCL21 and LIGHT is capable of inducing a synergistic antitumor effect[61].

Dendritic cell-based immunotherapy

Dendritic cells (DCs) are leukocyte populations that present antigens captured in peripheral tissues to T cells *via* both MHC class Ⅱ and I antigen presentation pathways[62]. DC maturation is referred to as the status of DC activation at which such antigen-presenting DCs leads to T-cell priming, while its presentation by immature DCs results in tolerance[63]. DC maturation is chiefly caused by biomolecules with microbial features detected by innate receptors (bacterial DNA, viral RNA, endotoxin, *etc*), pro-inflammatory cytokines (TNF, IL-1, IFNs), ligation of CD40 on the DC surface by CD40L, and substances released from cells undergoing stressful cell death.

It is well known that DCs are potent inducers of immune responses and the activation of these cells is a critical step for the induction of antitumoral immunity. We successfully tested a technique designed to take advantage of the therapeutic effect of IL-12 infecting DCs *ex vivo* with an adenovirus that expresses IL-12 genes (AdIL-12), and injecting the engineered cells into colorectal carcinomas in mice[64]. This strategy has proved to be exceptionally effective in eliminating neoplastic nodules and in eliciting anti-tumor immunity. This strategy is also effective in mouse models when DCs are transfected to express IL-7[65] and IL-15[66].

Transfection of DCs with mRNA is a promising antigen-loading technique of stimulating strong antitumor immunity. Chu *et al*[67] transfected RNA from CT26 colorectal adenocarcinoma to the bone marrow-derived monocytes and obtained strong specific CTL activity *in vivo*. Saha *et al* [68] showed that immunization of CEA transgenic mice with bone marrow-derived mature dendritic cells loaded with the antidote antibody 3H1 (which mimics CEA) resulted in a CEA-specific immune response and suppression of colon carcinoma cells (expressing CEA) in nearly 100% of mice, whereas only 40% of experimental mice immunized with dendritic cells loaded with CEA were protected from tumor growth.

Furumoto *et al*[69] injected MIP-3 chemokine together with CpGs into colorectal carcinomas in order to activate *in vivo* dendritic cells without *ex vivo* manipulation. These workers observed an increase in the number of activated DCs in tumors that were eradicated through specific T cell-mediated antitumor response.

CD40L, a costimulatory molecule expressed on activated $CD4^+$ T cells, acts on B cells and DCs, and plays a key role both for maturation of antibody responses and for CTL induction. Investigators from Crystal's group demonstrated in studies on mice, synergy in the eradication of subcutaneously implanted CT26 when treated with a combination of intratumor injection of an adenovirus expressing CD40-L with DCs or when each treatment was applied sequentially[70,71].

Morse *et al* reported a phase I clinical trial in which autologous dendritic cells loaded with carcinoembrionic antigen RNA (peptide CAP-1) were administered to patients with resected liver metastases from colorectal carcinoma. The procedure was well tolerated, and one patient had a minor response, and one showed stable disease[72]. With the aim to expand the presence of circulating DCs (DC mobilization), Fong *et al*[73] in a phase I study used the hematopoietic growth factor Flt3 ligand prior to the injection of CEA-derived peptide loaded DCs in 12 patients with colon or non-small cell lung cancer. One patient had a mixed response while two showed stable disease.

DCs engineered to produce IL-12 have been shown to induce potent anti-tumor responses. We have recently completed a phase I clinical trial which involved intratumor injection of monocyte-derived autologous dendritic cells transfected *in vitro* with an adenovirus encoding human IL-12 in patients with metastatic gastrointestinal carcinomas[46]. The main objectives of the trial were to assess feasibility and safety, and secondarily to determine biologic and clinical responses. We observed that this strategy was safe and well tolerated, with injection of up to 50×10^6 dendritic cells. Five patients showed increased NK activity and 4 showed augmented intratumor $CD8^+$ T-cell infiltrate. One partial response and two stabilizations were observed. The reasons for the weak antitumor response were explored. It appears that DCs can be retained inside malignant tissue by means of high intratumor concentrations of IL-8. Besides, scintigraphic tracking of intratumorally injected DCs labelled with ^{111}In indicated the retention of DCs inside malignant lesions in patients with digestive carcinomas[74].

CYTOKINE GENE TRANSFER FOR COLORECTAL CARCINOMA IN CLINICAL SETTING

Over 1100 gene therapy clinical trials have been carried out around the world and almost 70% of them were directed at the treatment of advanced or metastatic cancer. In clinical trials, cytokine and tumor antigen genes represent 42% of the genetic material that is transferred (for details see: www.wiley.co.uk/genmed/clinical). In the following section, we focus on some of the most important cytokines currently under clinical investigation in immunogene therapy of colorectal carcinoma.

The encouraging results obtained with the administration of non-replicative adenovirus encoding for IL-12 genes in several experimental models of gastrointestinal cancers (for review see reference[1])

prompted us to initiate a clinical trial at the University of Navarra in patients with advanced gastrointestinal carcinomas[46]. Patients with hepatic tumors (either primary or secondary colorectal carcinomas) were treated intratumorally in a dose-scale fashion with an adenovirus encoding human IL-12 genes. This strategy was safe and well tolerated with only minor side effects. Biological activity was observed in some patients (e.g., rise in serum levels of IFN-γ, infiltration of tumors by $CD8^+$ T cells and induction of neutralizing anti-adenovirus antibodies). Partial tumor regression was observed in one patient and stable disease in 30% patients. Reduction in the gap between doses in the same patient, or application of the vector as neoadjuvant therapy before tumor resection are some of the potential approaches to increase the efficacy of this treatment strategy.

The dose-limiting toxicity of large systemic concentrations of TNF- has led to a decline in its use in cancer patients. By contrast, local gene transfer of this cytokine using an adenovirus (TNFerade®) may reduce the systemic effects. TNF- gene under the control of an early growth response 1 (EGR-1) promoter followed by external beam radiation allows the control of TNF- release. Promising antitumor activity without any significant toxicity was observed in patients with solid tumors[75]. TNFerade® in combination with capecitabine and radiation therapy is now being tested in a phase Ⅱ clinical trial on patients with rectal cancer, before surgical resection.

Rubin *et al*[76] showed that direct gene transfer of HLA-B7 and 2-microglobulin, which together form a MHC-I complex, into the liver of patients with metastatic colorectal carcinoma is a feasible and safe procedure. These workers used a single plasmid construct that encodes for both genes in a formulation containing the lipid complex DMRIE-DOPE (Allovectin-7®). Genes transfected into tumors were detected by PCR in 14 out of 15 patients, however. the clinical results have not published. It should be noted that better results have been obtained in patient with melanoma.

With the advent of agents such as irinotecan and oxaliplatin, chemotherapy has made some progress in the treatment of colorectal carcinoma. The use of biological therapy with monoclonal antibodies against VEGF and EGFR has been shown to benefit a small proportion of patients[77]. Immunotherapy in different forms should be tested in addition to the conventional treatment regimens which improve patient survival.

Concluding remarks and future directions

There is a striking correlation between lymphocyte infiltration in colorectal cancer and the overall outcome of the disease[78,79]. Indeed, the density of T cells close to the tumor cells in the primary tumor is a better predictor of survival in these patients than traditional staging based on tumor size and spread[80]. According to this study, patients whose tumors contained large numbers of CD3-positive T cells, had a 5-year survival rate of 73%, compared with 30% in patients with low density of these cells.

There are important conclusions to be drawn from this study: (1) There is much natural immune pressure on colon cancer that may control the disease successfully in many patients, (2) The immune pressure possibly selects tumor variants that eventually escape immune control, (3) Artificial augmentation of the immune response may tilt the balance towards a curative response at least in some cases.

Immunotherapy intervention requires tumor-debulking and therefore should be combined with surgery and chemotherapy. To make the most of immunotherapy, this technique should be tested on patients whose tumors have been completely resected but are at high risk of relapse. For instance, our current efforts are focused on patients whose liver metastases have been resected surgically and are receiving adjuvant chemotherapy. In these patients, measures to induce/enhance cellular antitumor immune responses may confer a clinically significant delay in tumor relapse. Moreover, the complete removal of any detectable disease greatly diminishes the immunosuppressive mechanisms that may otherwise be induced by the cancer, while the surgical samples provide a rich antigenic source for immunization. Interference with the immunosuppressive mechanisms is clinically feasible with the use of low doses of cyclophosphamide[81] and other such mechanisms may become clinically available in the near future.

In our opinion, it is at the stage of minimal residual disease when immunotherapy should be fully deployed with a combination of strategies comprising of immunization with different tumor antigens and amplification techniques using cytokines or/and immunostimulatory monoclonal antibodies[82].

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TOPIC HIGHLIGHT

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Is there a genetic signature for liver metastasis in colorectal cancer?

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Abstract

Even though liver metastasis accounts for the vast majority of cancer deaths in patients with colorectal cancer (CRC), fundamental questions about the molecular and cellular mechanisms of liver metastasis still remain unanswered. Determination of gene expression profiles by microarray technology has improved our knowledge of CRC molecular pathways. However, defined gene signatures are highly variable among studies. Expression profiles and molecular markers have been specifically linked to liver metastases mechanistic paths in CRC. However, to date, none of the identified signatures or molecular markers has been successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice. To obtain a genetic signature for liver metastasis in CRC, measures to improve reproducibility, to increase consistency, and to validate results need to be implemented. Alternatives to expression profiling with microarray technology are continuing to be used. In the recent past, many genes codifying for proteins that are directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have been linked to mechanisms of liver metastases in CRC.

Key words: Colorectal cancer; Liver metastasis; Genetic signature; Expression profile; Arrays

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INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer, with a worldwide incidence of almost a million cases annually in both males and females[1]. Despite advances in screening, approximately 25% of patients have initially detectable liver metastases (synchronous metastases), and an additional 25% of patients will develop liver metastasis during the course of their disease (metachronous disease)[2]. Of all patients who die of advanced colorectal cancer (ACRC), 60% to 70% show liver metastasis[3]. Metastatic spread to the liver is the major contributor to mortality in patients with CRC.

CRC is a genetically heterogeneous and complex disease. Initially, two major pathways were described as being responsible for the CRC tumorigenic process: the chromosomal instability pathway and the microsatellite instability pathway. The chromosomal instability or classical pathway accounted for 85% of the tumorigenic processes and was mainly characterized by the sequential allelic losses on chromosomes 5q (APC gene), 17p (TP53) and 18q (DCC/Smad4). The microsatellite instability pathway (MNI), which is associated with the mutator phenotype, only accounted for 15% of the carcinogenic processes. Recently, it has been shown that colorectal carcinogenesis is much more complex, involving new pathways, such as the serrated, the TGFβ/Smad and epigenetic pathways, and also non-pure or mixed pathways[4-6].

The general mechanisms of tumorogenesis also include metastasis generation mechanisms. But, is the knowledge of CRC tumorigenic pathways extensible to metastasis generation? What do we really know about the molecular determinants of liver metastases formation in CRC?

MECHANISMS OF LIVER METASTASIS

Colorectal liver metastasis, or dissemination and colonization by colorectal tumor cells coming from the primary CRC to the liver, is a complex process and has many different steps. In order to metastasize, tumor cells detach from the primary tumor, invade and migrate through the stroma and intravasate into the lymphatic and/or venous vessels. With either as the vasculature entrance, cells will mainly end up travelling through the portal vein system. During transportation they manage to survive mechani-

Table 1 Summary of gene expression profile studies related to CRC liver metastasis

Source for transcription profile comparisons	Authors	Signature	Prediction
Primary tumors (Stage Ⅱ and Ⅲ)	Bertucci *et al*[11]	46 gene set	Lymph Node (+)
Primary tumors (Dukes C)	Arango *et al*[12]	Two different gene sets	Survival
Primary tumors (Stage Ⅱ and Ⅲ)	Barrier *et al*[13]	30 gene set	Lymph Node (+)
Primary tumors (Stage Ⅱ and Ⅲ)	Komuro *et al*[14]	Gene set	Stage Classification
Primary tumors (Stage Ⅱ and Ⅲ)	Kwon *et al*[15]	60 gene set	Lymph Node (+)
Primary tumors (Dukes B)	Wang *et al*[16]	23 gene set	Recurrence
Primary tumors (Stage Ⅱ to Ⅳ)	D´Arrico *et al*[17]	37 gene set	Distant Recurrence
Primary tumors and matched metastases	D´Arrico *et al*[17]	GnT-IV gene[1]	Liver Metastasis
Primary tumors and matched metastases	Koehler *et al*[18]	Not found	Liver Metastasis
Primary tumors and matched metastases	Agrawal *et al*[20]	11 gene set	Metastasis (including liver)
CRC cell lines[2]	Hegde *et al*[21]	11 gene set	Metastatic potential
CRC cell lines[2]	[11-14,16,17,22]	Individual genes[3]	Metastatic potential

[1]Mannosyl (alpha-1, 3-)-glycoprotein beta-1, 4-N-acetyl-glucosaminyl-transferase, which was found up-regulated in CRC liver metastases compared to primary CRC tumors; [2]Comparing SW480 to SW620; [3]Down-regulation of Cadherin 17 (CDH17)[11,22], Insulin-like growth factor 2 (IGF2)[14,17], Tyrosine 3-monooxygenase/tryptophan-5-monooxygenase activation protein (YWHAH)[12,16], DEK oncogene (DEK)[11,12] and GATA binding protein (GATA6)[11,14], up-regulation of Linker for activation of T cells (LAT)[14,16] and Protein Kinase, cAMP dependent, catalytic alpha (PRKACA)[12,14], and altered expression of IQ motif containing GTPase activating protein 1 (IQGAP1)[11,12], Tumor protein 53 (TP53)[11,12], Oligoadenylate synthetase 1 (OAS1)[11,12], Interferon regulatory factor (IRF2)[11,14], Retinoic acid receptor beta (RARB)[11,12] and Programmed cell death 10 (PDCD10)[12,13].

cal stresses and escape from the immune system. Some stresses keep acting once cells arrest in the liver capillaries. Some of the arrested cells manage to adhere to endothelial cells, contact the extracellular matrix and extravasate to the surrounding tissues. Kupffer cells, belonging to the monocyte-macrophage system, are a perfect barrier to unwanted hosts. Being in the liver parenchyma, tumor cells establish crosstalk with the stroma and create a microenvironment. Only if this microenvironment is favourable to tumor cells, signals of proliferation and neoangiogenesis will lead to macroscopic liver metastasis formation[7-9]. Even though liver metastasis accounts for the vast majority of all cancer deaths in patients with colorectal cancer, fundamental questions about the molecular and cellular mechanisms of liver metastasis still remain unanswered.

Genetic signatures: The breakthrough

The availability of DNA array technology, allowing genome-wide analyses of gene expression, has been providing new insights on the determination of gene expression or transcriptional profiles. Expression profiling studies in CRC have mainly focused on comparisons of normal mucosa, adenoma and primary carcinomas. Few studies have thrown light on differences between primary tumors and metastases. For this reason, in contrast to the many molecular alterations involved in the CRC adenoma to carcinoma step characterized to date, comparatively little information is available on the possible mechanisms of metastases, with even less for liver specific metastases[10].

There are two different aspects of metastasis to consider: metastatic ability and tropism or organ-specificity. Metastatic ability accounts for the potential to establish a distant secondary tumor. Organ-specificity or tropism means the capacity of this happening in a specific type of tissue. The ability to metastasize together with the specificity for it to happen in one organ and not in another can be genetically marked by what is called a metastatic signature. Studies looking at mRNA or protein levels take into account expression regulation, splicing mechanisms, epigenetic phenomena, and the complexity of post-translational changes or modifications. A metastatic signature, therefore, is not a gene list but is a translation of the functional status of gene expression. Metastatic signatures are gene expression patterns conditioned by both an intrinsic gene composition and phenomena regulating expression.

In order to determine metastatic signatures by microarray technology in CRC, three different strategies have been followed (Table 1). The first approach consists of comparing transcriptional profiles of primary CRC from metastasis-free patients to those affected by metastatic spread during a 5-year follow-up period. The main goal is finding gene expression profiles as prognostic markers of metastatic spread. Identification of a gene set capable of classifying CRC patients according to prognosis or 5-year survival rate was carried out by Bertucci *et al*[11]. A total of 219 genes and 25 genes were found to be respectively down- and up-regulated in metastatic samples when compared to non-metastatic patients. Moreover, a 46 gene set signature was isolated, discriminating between CRC with and without lymph node metastases. Arango *et al*[12] checked the expression profile of Dukes C CRC and reported two different signatures according to survival. Barrier *et al*[13] built an accurate 30-gene tumor-based prognosis predictor for stage Ⅱ and Ⅲ colon cancer patients, based on gene expression measures. The group of Komuro *et al*[14] analyzed gene expression profiles in a total of 89 CRC. After stratifying according to right and left locations, they were able to extract gene expression profiles characteristic of the presence versus absence of lymph node metastasis with an accuracy of more than 90%. Kwon *et al*[15] analyzed the gene-expression profiles of colorectal cancer cells from 12 tumors. Sixty genes possibly associated with lymph node metastasis in CRC were selected on the basis of clinicopathological data. Wang *et al*[16] analyzed RNA samples from 74 patients with Dukes' B CRC. Gene expression profiling identified a 23-gene signature that predicted recurrence. This signature was validated in 36 independent patients. The overall

performance accuracy was 78%. D´Arrico *et al*[17] compared the transcriptional profiles of 10 radically resected primary CRCs from patients who did not develop distant metastases within a 5-year follow-up period with those of 10 primary/metastatic tumor pairs from patients with synchronous liver metastases. The study was conducted on laser-microdissected bioptic tissues. Arrays of 7864 human cDNAs were utilized. Non-metastasizing primary tumors were clearly distinct from the primary/metastatic tumor pairs. Of 37 gene expression differences found between the 2 groups of primary tumors, 29 also distinguished nonmetastasizing tumors from metastases. The gene encoding for mannosyl (alpha-1, 3-)-glycoprotein beta-1, 4-N-acetyl-glucosaminyl-transferase (GnT-IV) became significantly upregulated in primary/metastatic tumor pairs ($P < 0.001$), supporting the existence of a specific transcriptional signature distinguishing primary CRCs with different metastatic potential[17].

The second approach consists of comparing gene expression in primary tumors with their matched metastases. Studies comparing gene expression between primary and corresponding metastases indicate that there is a high transcriptional resemblance. The above mentioned study found a striking transcriptional similarity between primary tumors and their distant metastases[17]. Another study by Koehler *et al*[18] determined expression profiles from 25 CRCs and 14 corresponding liver metastases using cDNA arrays containing 1176 cancer-related genes. Most primary tumors and matched liver metastases clustered together. A specific expression signature in matching metastases was not found, but a set of 23 classifier genes with statistically significant expression patterns in high- and low-stage tumors was identified. Gene expression studies in breast cancer also support the notion that primary tumors genetically resemble their matched metastases more than their primary counterparts[19]. Agrawal *et al*[20] found a signature of 11 markers for tumor progression when comparing gene expression among different stages, including liver metastases in a total of 60 samples.

Expression profiling using CRC cell lines with different metastatic potential is another approach[21,22]. Studies using cDNA microarrays have identified genes that are differentially expressed in primary *versus* metastatic CRC cell lines. Differential expression of 11 genes has been found in SW480 and SW620 CRC cell lines[21]. Unfortunately, metastatic signatures described in the above mentioned studies do not show much in common. Gene expression patterns do not overlap enough to show consistency. Only a few genes reported in at least two independent studies have been linked to metastatic ability (Table 1).

It is interesting that no expression profile has been specifically linked to liver metastases in CRC. Apart from gene expression profiling, other techniques, such as genomic profiling, have also been used to determine metastatic ability in CRC. Genomic analyses of primaries and their matched metastases[23] showed that CRC primary tumors resemble their corresponding metastases. Array-based comparative genomic hybridization (CGH) was used to detect genetic alterations in CRC that predicted survival after liver resection[24]. Genome wide copy number analysis revealed the involvement of Cycline D3 in liver metastases formation in CRC[25].

Genetic signatures: Handicaps and pitfalls

When determining metastatic expression profiles or signatures with array technology, several confounders have to be taken into account. Studies have employed important methodological differences, which are mainly due to the use of different array platforms (Affymetrix, cDNA nylon membranes) or experimental conditions. Tissue sampling is almost always an issue in this regard. Availability of frozen tissues is not the norm in many institutions. Formalin-fixed or paraffin-embedded tissues usually yield low quality RNA and/or DNA. The creation of frozen-tissue tumor banks is rapidly increasing. Also methodologies for RNA isolation can lead to different results. The number of samples used varies enormously in different studies. Relatively small cohorts of tumors have been analyzed in some studies, especially if they include the analysis of matched metastases. Selection of homogeneous samples among heterogeneous tumors can often be a problem. Anatomical localization (right *vs* left sided, colon *vs* rectum) and genetic instability status (MSI/classical) may justify the variability of CRC gene expression profiles characterized to date. Macrodissection techniques include tumor tissue with both tumor cells and tumor stroma and valid tissue samples should be at least 50% tumor cells. One of the major criticisms of "metastatic signature" -seeking studies is the fact that tumors are analyzed as a whole, mixing tumor cells with microenvironment and stroma components. Certainly, data coming from these experiments is a mixture representing gene expression of tumor cells, stroma cells as well as their interactions. Moreover, expression data can be highly conditioned by the host genetic background. Resulting data can be highly interesting in terms of defining prognosis, but not in understanding the mechanisms of metastasis generation. Microdissection techniques help to avoid this problem. Laser capture microdissection (LCM) allows isolation of only tumor cells and is considered the gold standard in microdissection procedures[26]. It is a time-consuming technique and it is not available at all institutions. Other strategies include subtracting non-tumor cell signatures from gene expression data[27]. It is still unclear whether the analysis of pure tumor cell populations will lead to an appropriate result in terms of prognostic value.

Description of metastatic signatures has been done on the basis of transcription analysis of tumors. Data from DNA microarray analysis is often overwhelming and mixed. Analysis of differentially expressed genes can be altered by the use of different criteria to define low-quality spots, different normalization procedures, different baseline references for ratio calculations, and arbitrary criteria for cut-off values applied to fold-change and significance level. Commonly, quantitative levels of expression are the basis for filtering the raw data. During filtering, information coming from qualitative data can be lost[10]. Moreover, the final data set has to be interpreted and integrated to make sense in biological terms. This step is highly subjective and probably often leads to false conclusions. Nearly

Table 2 Proteins related to liver metastasis formation and their function, and their differential expression when comparing primary tumors and liver metastasis by immunohistochemical technique

Proteins related to liver metastasis formation	Function	Liver expression compared to primary tumor (IHC)
E-Cadherin	Adhesion	Down-regulated[34]
Epithelial Cell Adhesion Molecule (EpCAM)	Adhesion	NA
P-Selectin and L-Selectin	Adhesion	NA
Carcinoembryonic Antigen (CEA)	Adhesion	NA
Integrin αvβ5	Adhesion, Survival	NA
sLex and sLea	Adhesion	Up-regulated[48,51]
Osteopontin (OPN)	Adhesion, Survival, Motility	Up-regulated[63]
Intracellular Adhesion Molecule (ICAM-1)	Adhesion	NA
Vascular Cell Adhesion Molecule (VCAM-1)	Adhesion	NA
CD44v6	Adhesion	NA
Cathepsine B	Invasion	NA
MMP-7	Invasion	Up-regulated[81]
MMP-2 and MMP-9	Invasion	Up-regulated[86]
Angiopoietin	Angiogenesis	Up-regulated[110]
Epidermal Growth Factor Receptor	Growth	Equal[125]
Urokinase Plasminogen Activator Receptor (uPAR)	Invasion, Motility, Dormancy	NA
Vascular endotelial Growth Factor (VEGF)	Angiogenesis	Equal[109]
Thrombospondin-1 (TSP-1)	Angiogenesis	NA
Angiostatin	Angiogenesis	NA
Endostatin	Angiogenesis	NA
Thimidine Phosphorylase (dThdPase or PDECGF)	Angiogenesis	NA
c-erb-2	Growth	NA
c-Src/β-Arrestin 1	Growth	NA
FAS Receptor (CD95)	Apoptosis	Down-regulated[134]
TRAIL Receptors (-R1, -R2, -R3 and -R4)	Apoptosis	NA
Nm23-H1 and Nm23-H2	Metastasis Suppresor Genes	NA
PRL-3	Motility, Extravasation	Up-regulated[157]

NA: Not available.

all studies lack internal and external validation tests for the generated lists of implicated genes. Different selection algorithms should be tested in order to improve the accuracy of the classifier sets[10].

In conclusion, to obtain a genetic signature for liver metastases in CRC, measures to improve reproducibility, increase consistency, and validate results need to be implemented.

Genes involved in liver metastasis formation in CRC

Alternatives to expression profiling by microarray technology have also been used in recent past years. Many genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have been linked to mechanisms of liver metastasis in CRC[28] (Table 2).

Adhesion: Different proteins involved in adhesion/deadhesion processes have been linked to liver metastasis development in CRC. Deadhesion is a necessary step for tumor cells to detach from a tumor and disseminate. Adhesion is needed for circulating cells to contact helping counterparts in the dissemination process. It is also needed to attach to the vascular endothelium, induce endothelial retraction, and subsequently bind to glycoproteins of the basement membrane to extravasate.

E-cadherin/α-catenin is a cell to cell adhesion complex that keeps tumor cells together. Cells detaching from the primary CRC undergo an epithelial to mesenchymal transition, during which E-cadherin downregulatates in favour of other cadherins, such as N-cadherin. This process is known as the "cadherin switch" and leads to acquisition of a mesenchymal phenotype that favours invasion and migration through the stroma and thus dissemination of tumor cells[29]. Downregulation of E-cadherin/α-catenin expression has been related to tumor aggressiveness[30,31] and metastatic potential[32,33] in gastrointestinal cancers. Low expression of α-catenin and E-cadherin in CRC patients has been associated with an increment of β-catenin[34-36], advanced stages[33,37,38] and acquisition of metastatic potential[39,40]. Immunohistochemical studies show that CRCs metastasizing to liver have a significant ($P = 0.014$) reduction or complete absence of E-cadherin expression when compared to non-liver metastases[34].

Epithelial cell adhesion marker (EpCAM) is a widely expressed adhesion molecule. It has been found to present a more diffuse pattern and higher expression in CRC compared to non-malignant tissues[41]. EpCAM plays a role in modulating cadherin mediated cell-cell interactions[42] and its expression has been linked to downregulation of cadherin levels[43], suggesting that this protein possibly plays a role in ETM processes, facilitating migration and dissemination of tumor cells. Supporting this notion, isolation of EpCAM positive cells in blood samples of advanced CRC patients[44] has recently been achieved. All these preliminary data suggest that possibly EpCAM plays a role in CRC cell dissemination. Whether there is liver specificity remains unknown.

Sialyl Lewis X (sLex or CD15s) and A (sLea) are oligosaccharides commonly found in surface glycoproteins

of metastatic tumor cells[45]. sLex and sLea are natural ligands for E-selectin, which is a receptor that has been found to be expressed by activated endothelial cells. Interaction between sLex and sLea induces endothelial adhesion of tumor cells and thus favours stasis, extravasation and metastases formation. sLex and sLea expression in primary CRC have been related to poor prognosis[46] and metastatic potential[46-48] in CRC patients. sLex and sLea stain significantly positive in vessel invasion CRC cells that develop metastases compared to those that do not (71.4% *vs* 31%)[49]. sLex and sLea have been found to be present on the surface of tumor cells[50] in CRC patients who develop liver metastases. Similarly, CRC liver metastases express sLex and sLea in a larger proportion of tumor cells than in primary tumors[48,51]. E-selectin is overexpressed by endothelial cells from tumor and non-tumor vessels in CRC patients who develop liver metastasis[52,53]. In general, as has been demonstrated in *in vivo* models, glycosylated and sialylated mucins are associated with liver metastasis formation[54]. Some proteins allow the adhesion of CRC cells with blood components, such as platelets and leukocytes. Among those proteins are P-Selectin and L-Selectin. This interaction facilitates tumor emboli formation, favouring protection of tumor cells from immune attack and also enhancing their ability to contact blood vessels by mechanical means. This interaction between tumor cells and blood cells also increases contact with the endothelial surface, facilitating stasis and thus enhancing the chances of extravasation[55].

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein containing significant amounts of sLex and sLea. Expression of (CEA) has been clearly correlated to generation of liver metastases in experiments transfecting CEA to CRC cell lines or administering CEA in animal models previous to CRC cell injection[56]. Initially it was speculated that CEA would act as an adhesion molecule, facilitating tumor cell aggregation and interaction with the endothelial surface. However, studies with immunosuppressed mice show that administration of intravenous CEA results in an increase of hepatic colonization and retention of CRC cells, but not an increase of adhesion[57]. Kupffer cells that express a CEA receptor bind to and degrade it, activating a signaling cascade that ends up releasing IL-1, 6 and TNF-α which, in turn, facilitates CRC cell stasis and growth[58,59]. The ability to secrete CEA offers CRC cells a selective advantage in forming metastases in the liver.

Integrins are molecules that can bind to many ECM components, such as laminin, collagen, fibronectin and vitronectin. Cancer cells expressing integrins are more likely to adhere to ECM components surrounding microvasculature. High expression of $\alpha 6\beta 4$ and $\alpha 5\beta 3$ integrins has been related to a more aggressive CRC phenotype[60,61]. Intravital fluorescence-video microscopy has been used to investigate liver metastas formation by CRC cells in animal models[62] and results have shown that $\alpha v\beta 5$ integrin is useful as an adhesion molecule and its inhibition diminished liver metastas formation.

Osteopontin (OPN) is a secreted phosphoglycoprotein capable of binding and inducing integrin-mediated cell survival, motility and anti-apoptotic intracellular pathways. OPN has been isolated in gene expression profiling studies as a candidate marker for CRC progression[20]. CRC liver metastases express OPN at higher ratios than primary CRC or normal mucosa[63]. OPN up-regulation can occur due to TCF4/LEF transcription factor activation[64]. Mechanisms by which OPN promotes liver metastases formation in CRC are unknown, but could be related to up-regulation of Upa[65], c-Met receptor and integrins[66].

Other adhesion molecules, such as the intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), have been measured in ACRC patients showing higher serum levels when compared to non-advanced CRC or healthy controls[67,68]. Nevertheless, neither clinical nor physiological relation has been established with specific development of liver metastases.

CD44 glycoprotein, more specifically v6 and v8-10 splicing variants, have been related to metastases and disease recurrence in CRC[69,70]. There is quite a bit of controversy regarding the real value of CD44 in liver metastases formation because plasma levels have not been linked to advanced stages of the disease[71] and immunohistochemical studies measuring CD44v6 staining have not found significant differences when comparing CRCs metastasizing to liver or not[34].

Invasion: Invasion processes are crucial for liver metastasis formation in CRC. Invasion occurs mainly due to basal membrane and extracellular matrix (ECM) degradation in both intravasation and extravasation steps. Some of the enzymes responsible for degradation are proteases. Among proteases, matrix metalloproteases (MMPs), cathepsins and plasminogen activators are the most relevant.

Matrylysin (MMP-7) is a proteolytic enzyme belonging to the MMPs family[72,73]. It is synthesized and secreted by tumor epithelial cells as a 28-kDa proenzyme, which can be activated through proteolytic removal of a 9-kDa prodomain from the N-terminus. The soluble activated form binds to the tumor epithelial cell surface. Both active forms, the soluble and the membrane-bound, have proteolytic activity. Its expression can be regulated by epidermal growth factor through transcription factors such as PEA3[74] or AP-1 and the β-catenin/ tcf4 complex. By degrading elastin, laminin, proteoglycans, osteopontin, fibronectin and type Ⅳ collagen, MMP-7 gains the capacity to invade. Matrilysin can also promote tumor invasion by activating other MMPs (MMP-2, MMP-9), through ectodomain shedding of E-cadherin[75] and receptor activator of nuclear factor-kappa B ligand (RANKL)[76] or through cleavage of adhesion molecules, such as integrin $\beta 4$[77].

Matrilysin has been found overexpressed in CRC[78]. MMP-7 overexpression in localized CRC disease has been correlated with invasion and liver metastasis formation[79,80]. Colorectal liver metastases show intense expression of MMP-7 compared to normal liver, and differences are more evident when comparing the MMP-7 activated form, measured by zymography, emphasizing the role of MMP-7 in CRC liver metastases formation[81]. While testing liver metastasis formation *in vivo*, it has been shown that treating colorectal cancer cells with MMP-7 specific antisense

oligonucleotides leads to a decrease in liver metastasis generation[82], while adding active MMP-7 results in an increase of liver metastasis generation[83].

MMP-9 and MMP-2 also seem to play a role in liver metastasis formation in CRC. High MMP-9 and MMP-2 levels have been detected by immunohistochemistry in the tumor-stroma interface in both primary CRC and liver metastases[84,85]. Moreover, MMP-2 and -9 activities seem to be higher in metastases than in the originating primary tumor[86]. A close correlation between high MMP-9 RNA levels and worse survival and higher risk of liver relapse after surgery has also been established[81].

Cathepsins have also been implicated in liver metastasis formation in CRC. They are a family of proteolytic enzymes with a wide variety of physiological functions. They act as serin-proteases, cystein-proteases or aspartate-proteases. They are stored as proforms in cell lysosomes and secreted to the ECM secondarily to inflammatory and oncogenic stimuli[87].

Cathepsins B, L and D are especially involved in ECM degradation in CRC. Their levels and activity[87-88] have been found to be elevated in the invasion edge of CRC. Still, Cathepsin B is the most valuable in determining invasion in CRC[89]. Cathepsin B degrades ECM directly or indirectly, by stimulating other proteases or blocking their inhibitors[87]. It can be detected in early stages of CRC but it is a good marker to determine metastatic disease[90,91]. High plasma and urine levels of Cathepsin B have been found in CRC patients[92]. *In vivo* experiments show that inhibition of Cathepsin B by selective compounds results in reduction of liver metastases formation up to 60% and reduction of liver metastases burden up to 80%[93]. A proteolytic profile, taking into account MMP and cathepsin expression, has been defined for CRC by some authors[94].

Urokinase plasminogen activator receptor (uPAR) is a factor involved in metastasis development in several cancers[95,96]. uPAR binding to urokinase plasminogen activator (uPA) enhances plasmin production which, in turn, degrades ECM and activates pro-MMPs. Inhibition of uPAR expression is associated with decreased motility and invasiveness in the human CRC cell line HCT116[97]. High uPAR expression in CRC has been related to low 5-year survival[98]. Use of antisense uPAR mRNA in a nude mice model inhibited CRC liver metastasis development[99].

During invasion, apart from basal membrane and ECM degradation processes, cancer cells have to migrate through the stroma. Clues for success are acquisition of a mesenchymal phenotype during ETM and ability to survive independently of the tumor cell population. To gain the ability to disseminate, tumor cells have to detach from the tumor population, overcome anoikis and transit from an epithelial to a mesenchymal phenotype. As a principle, cells need to be in contact with other cells in order to survive. If they lose contact or penetrate to the ECM they undergo anoikis. Overcoming anoikis, an apoptotic program related to tumor cell population detachment, is a necessary requirement to disseminate. Integrins are responsible for epithelial cancer cell cross-talk with the ECM in order to overcome anoikis, survive and migrate.

In vitro experiments have shown that activation of Src and Akt pathways are linked to decreased sensitivity of detached CRC cells to anoikis[100]. Down-regulation of $\alpha v\beta 3$ integrin has also been linked to resistance to anoikis in CRC cells[101,102]. Integrins can bind to many ECM components such as laminin, collagen, fibronectin and vitronectin. Cancer cells expressing these integrins are more likely to invade and migrate through the ECM[103,104]. High expression of $\alpha 6\beta 4$ and $\alpha 5\beta 3$ integrins has been related to more aggressive CRC phenotypes[60,61]. Intravital fluorescence-video microscopy has been used to investigate liver metastasis formation by CRC cells in animal models[62] showing that $\alpha v\beta$-integrin inhibition did not affect migration within the liver parenchyma. The role of integrins in the migration and invasion through the ECM in order to generate liver metastasis has not been extensively explored.

Angiogenesis: Different angiogenic factors have been related to metastasis formation because they can promote primary tumor growth and increase tumor cell chances to contact blood and thus disseminate. However, it is likely that angiogenesis plays a major role in metastasis generation regulating micrometastases outgrowth. Balance between angiogenic/antiangiogenic factors in the microenvironment of the metastatic tissue can promote metastasis formation by directly stimulating tumor cell growth or by increasing blood vessel formation and supply. Even in quiescent tumor cells, alteration of angiogenic balance can induce metastasis formation. This phenomenon is known as "angiogenic switch"[105] and causal factors are still under investigation.

Expression levels of vascular endothelial growth factor (VEGF) in primary CRC have been related to a poor prognosis[106]. VEGF isoform patterns have been defined using reverse transcription polymerase chain reaction (RT-PCR) analysis in 61 primary CRC. Patients developing liver metastases showed expression of VEGF121 + VEGF165 + VEGF189 at a significantly higher incidence (12 of 16, 75%) than those without liver metastasis (20 of 45, 44%) ($P = 0.036$)[107]. VEGF expression in primary CRC seems clearly associated with increased chances of dissemination. However, other studies support the contrary[108]. When VEGF mRNA levels were measured in 31 pairs of primary CRC and corresponding liver metastases, no significant differences were detected (median value 3.79 *vs* 3.97: $P = 0.989$). On an individual basis, there was a significant correlation in VEGF mRNA expression between primary CRCs and matched liver metastases ($r = 0.6627$, $P < 0.0001$). VEGF mRNA levels of patients having two or more liver metastatic tumors were significantly higher than those of patients who had solitary liver metastatic tumors in both primary cancer (5.02 *vs* 3.34: $P = 0.0483$) and liver metastases (4.38 *vs* 3.25: $P = 0.0358$)[109]. Together these results indicate that VEGF is probably not more active in metastases than in primary tumors. Despite that, increased blood supply and tumor vessel formation, as estimators of angiogenic activity, have been found to be higher in liver metastases that in primary CRC. Some molecular mediators have been thought to fulfill this role, such as angiopoietin-2 (Ang-2)[110].

Other distinctive molecules related to angiogenesis and

liver metastatic progression are platelet-derived endothelial cell growth factor or thymidine phosphorylase (PD ECGF or dThdPase). Inhibitors of angiogenesis, such as angiostatin, endostatin and thrombospondin-1 (TSP-1), either secreted by the primary or the metastatic CRC cells, can also regulate liver metastasis growth. Frequency of hepatic recurrence was significantly higher in patients with TSP-1-negative primary CRC[111]. Angiostatin transfected cells developed liver metastases in lower proportion than controls in animal models[112]. Removal of primary CRC resulted in an increase in metabolic activity in liver metastasis, while decreases in plasma levels of angiostatin and endostatin were observed. This finding indicates that primary tumors suppressed angiogenesis in distant metastases, and that removal of the primary lesion caused a flare-up in vessel neoformation and, thus, enhanced metabolic activity in liver metastases[113].

Other molecules mentioned above also contribute to liver metastasis formation through angiogenesis regulation. MMP-7 induces a direct proliferative effect on vascular endothelial cells[114] and produces angiogenesis inhibitors (angiostatin, endostatin, neostatin-7)[115] and activators (sVEGF)[116]. MMP-2 and MMP-9 stimulate degradation of ECM, increasing the availability of angiogenic activators. E-selectin acts by facilitating endothelial cell migration. α and β integrins play an important role by sending survival signals for endothelial cell maintenance[117].

Cell growth: Once established in the liver tissue microenvironment, micrometastases need growth factor stimuli in order to grow. Degradation of ECM results in an increased availability of growth and inhibitory factors. The resulting balance will then determine micrometastasic growth. Extrapolation to a non-physiological situation can be highly illustrative. Liver tissue thermal ablation was performed in mice models bearing CRC liver metastases. After ablation, increased expression of FGF-2 and VEGF was detected in the surrounding tissue. Subsequently, a greater amount of metastases occupied the regenerated thermal-ablated lobe compared with controls (55% ± 4% *vs* 29% ± 3%, $P < 0.04$)[118].

Tumor cells growth factor receptors also seem to determine success in metastatic liver growth. Her-2/neu has been detected by immunohistochemistry in 5% to 50% of primary CRC[119]. The mechanism of overexpression seems to be not linked to gene amplification. Her-2/neu positive CRCs were associated with higher postoperative non-liver specific recurrence rates (39.3% *vs* 14.6%, $P = 0.013$) and worse prognosis at 5 years (55.1% *vs* 78.3%)[120]. Other studies showed that primary CRC with high c-erbB-2 expression (27%), determined by immunohistochemical techniques, develop liver metastases more often than CRC with low c-erb-2 expression (3%)[49].

Epidermal growth factor receptors (EGFR) have been reported to be highly expressed and/or gene amplificated in 72% to 82% of metastatic CRC tissue samples[121-123]. Some studies have reported that expression of EGF receptors in CRC is associated with aggressiveness and metastatic ability. EGFR status has been shown to express similarly when measured in primary CRC and corresponding liver metastases[124]. However, some authors have seen that its status in the corresponding metastatic site is not always the same[125,126]. Conventional immunohistochemistry techniques have not been able to reveal any association between EGFR expression and outcome predicted by the biological role of EGFR in tumor behavior[127].

The C-Src gene, codifying for pp60 tyrosine kinase, has been reported to be mutated and thus is highly activated in CRC, implying an increase in proliferative potential. High activation is present especially in those CRC that metastasize to liver[128,129]. Prostaglandin E_2 (PGE_2)-induced transactivation of the EGF receptor (EGFR) in colorectal carcinoma cells has been recently found to be mediated by β-arrestin 1, which acts as an important mediator in G protein-coupled receptor-induced activation of c-Src. Interaction of beta-arrestin 1 and c-Src seems to be critical for the regulation of CRC metastatic spread of disease to the liver *in vivo*[130].

Cell survival: CRC cells need molecular factors, specifically growth factors, in order to survive in the liver parenchyma. However, there is also the need to survive immune system action (immunoescape) and to overcome anoikis.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is known to be expressed in human hepatic NK cells[131]. CRC cells expressing TRAIL-receptor would undergo apoptosis upon triggering the ligand. The same would happen in CRC cells expressing tumor necrosis factor receptor FAS (Apo-1; CD95) when contacting its corresponding ligand FASL (Apo-1L; CD95L) expressing cells, as activated lymphocytes.

During the CRC tumorigenic process, cells tend to down-regulate FAS receptor expression and up-regulate FASL[132]. Fas expression is significantly down-regulated in liver metastasis compared to corresponding primary colorectal carcinoma[133]. The link between functional Fas status and malignant phenotype was investigated using matched pairs of naturally occurring primary (Fas-sensitive) and metastatic (Fas-resistant) human colon carcinoma cell lines in both *in vitro* and *in vivo* (xenograft) settings. Results showed that loss of Fas function was linked to the acquisition of a detectable metastatic phenotype, however, only loss of Fas function was insufficient. Also, results showed that metastatic subpopulations pre-existed within the heterogeneous primary tumor and that anti-Fas interactions served as selective pressure for their outgrowth. Thus, Fas-based interactions may represent novel mechanisms for the biological or immunological selection of certain types of Fas-resistant neoplastic clones with enhanced metastatic ability[134]. Moreover, univariate and multivariate analyses revealed that Fas/CD95 expression in CRC resected liver metastases is a significant prognostic indicator of survival[135]. Increases in TRAIL sensitivity, due to changes in the balance between TRAIL receptors TRAIL-R1 and -R2 and "decoy" receptors TRAIL-R3 and -R4, have also been described during malignant progression in CRC. Still, studies measuring receptors by flow cytometry have not

been conclusive[136].

Experimental metastases studies with a CRC cell line allowed the characterization of metastatic derivatives, showing that they were less susceptible for killing by syngeneic NK cells, due to a decreased sensitivity towards TRAIL- and CD95L[137]. Data suggest that CRC cells forming metastases acquire the ability to surpass immune surveillance through desensitization to FAS/TRAIL killing. As discussed previously, integrins and Src activation may contribute to CRC progression and liver metastasis, in part, by activating survival pathways that decrease sensitivity of detached cells to anoikis[100].

Other molecules related to liver metastatic spreading: k-ras (12p) activation, present in 40% to 50% of sporadic CRC[4], has been related to a decrease in overall survival and disease free survival in CRC[6,138,139]. p53 (17p) abolition, occurring in 70% to 80% of CRC[4] and resulting in accumulation of abnormal protein detectable by immunohistochemistry, has been linked to a poor prognosis[6,140-142]. The deletion or mutation of the DCC (deleted in colorectal cancer) gene has also been related to poor prognosis tumors[143-146]. Even p53, Ras and/or DCC alterations have been linked to metastatic spreading in CRC, however, there is still no evidence specifically relating them to liver metastasis formation. The human nm23 genes, nm23-H1 and nm23-H2, are candidate metastasis suppressor genes. Their role in CRC is still confusing. Some authors claim that a reduced protein expression, secondary to gene alterations, is associated with metastasis development[147,148]. Genetic alterations were detected in four of eight CRCs associated with metastasis in lymph nodes, lung, or liver, while no alteration was observed in 12 additional CRC specimens without metastasis[149]. Others have found that gene overexpression is linked to higher recurrences, liver metastasis and decreased overall survival[150,151]. This contradiction could be explained if overexpression of nm23 was a reflection of a deletion in the nm23 gene, leading to accumulation of an altered protein product. However, more recent works have not been able to relate nm23 expression to prognosis[152-154]. The PRL-3 protein tyrosine phosphatase gene gained importance in 2001 when an article was published in Science showing that it was expressed at high levels in each of 18 cancer metastases studied but was expressed at lower levels in nonmetastatic tumors and in normal colorectal epithelium[155]. Subsequently, new data established an unexpected and unprecedented specificity in metastatic gene expression profiles: PRL-3 was apparently expressed in CRC metastasis to any organ but was not expressed in metastases of other cancers to the same organs or in nonmetastatic CRC[156]. At that time PRL-3 was determined to be a potential marker for liver metastasis of CRC with a negative impact in prognosis[157]. CRC specificity was objected to in further studies. Some authors claimed that PRL-3 acted by enhancing cell motility and thus facilitating extravasation into liver tissue[158]. The mechanism of action is still under investigation but it has already been related to integrin $\alpha 1$[159] and the Rho family of small GTPases[160].

CONCLUSION

A significant amount of experimental data points to tumor cells having a metastatic signature. This signature codifies not only for the ability to form metasteses but also for organ-specificity. DNA microarray technology has significantly improved efficiency in wide-range analysis of gene expression. Many authors have provided gene expression profiles that have been related to CRC liver metastases, however, in order to obtain a real genetic signature for liver metastases in CRC by transcription profiling, measures to improve reproducibility, increase consistency, and validate results need to be implemented. Seeking metastatic signatures through expression profiling is a tool to fight cancer, but its indiscriminate use can be misleading. Advances in molecular assays on isolated cells and in the study of cell-cell and cell-stroma interactions will likely enable the dissection of the metastatic cascade. Genes codifying for proteins directly or indirectly involved in adhesion, invasion, angiogenesis, survival and cell growth have already been linked to mechanisms of liver metastases in CRC. Improvement in knowledge of the molecular pathways involved in the development of colorectal liver metastasis will lead to a better approach to prevent and treat this disease.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Exploiting novel molecular targets in gastrointestinal cancers

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Abstract

Novel molecular targets are being discovered as we learn more about the aberrant processes underlying various cancers. Efforts to translate this knowledge are starting to impact on the care of patients with gastrointestinal cancers. The epidermal growth factor receptor (EGFR) pathway and angiogenesis have been targeted successfully in colorectal cancer with cetuximab, panitunumab and bevacizumab. Similarly, EGFR-targeting with erlotinib yielded significant survival benefit in pancreatic cancer when combined with gemcitabine. The multi-targeting approach with sorafenib has made it the first agent to achieve significant survival benefit in hepatocellular carcinoma. Efforts to exploit the dysregulated Akt/mTOR pathway in GI cancer therapy are ongoing. These molecular targets can be disrupted by various approaches, including the use of monoclonal antibody to intercept extracellular ligands and disrupt receptor-ligand binding, and small molecule inhibitors that interrupt the activation of intracellular kinases.

Key words: Colorectal; Pancreatic; Liver cancers; Targeted therapy; Epidermal growth factor receptor; mTOR; Angiogenesis

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INTRODUCTION

Cellular proliferation, differentiation and death are regulated by a number of extracellular factors, such as hormones, cytokines and growth hormones. Interactions between extracellular stimuli and the nucleus is mediated by a complex and interconnecting network of signaling pathways[1]. This process is often abnormal in cancer cells and our understanding of these molecular events led to the identification of novel targets for therapy development. Various approaches are been used to target these dysfunctional elements, including ligand neutralization, disruption of receptor binding, and inhibition of receptor kinases and intracellular signal messengers.

A plethora of compounds are now under development that targets these aberrant processes. Almost all of these biological agents have limited single agent activity but are synergistic when combined with conventional cytotoxic agents[2]. Therefore, they are usually tested in combination with standard therapy in specific cancer types. In colorectal cancers, fluorouracil-based regimens form the backbone of therapy in both adjuvant and metastatic settings[3-5]. Likewise, gemcitabine based therapy remains the cornerstone for untreated advanced pancreatic cancer and sorafenib is likely to become the standard therapy for hepatocellular carcinoma (HCC)[6-8].

Successful targeting of angiogenesis and the epidermal growth factor pathway has made colorectal cancer a prototypical model for the development of signaling pathway-specific agents in gastrointestinal (GI) cancers[9-11]. Akt/mTOR pathway is another candidate target in anti-cancer therapies[12]. This paper will review the approaches currently used to exploit these novel targets in the development of GI cancer therapy. The review will focus specifically on colorectal, pancreatic and primary liver cancers (hepatocellular carcinoma, or HCC).

EPIDERMAL GROWTH FACTOR RECEPTOR PATHWAY

Epidermal growth factor receptor (EGFR) is a member of the HER-family kinases, which includes EGFR, HER2, Erbb3 and Erbb4[13,14]. Upon ligand binding, EGFR homodimerizes with another EGFR or other members of the HER-family (heterodimerization), and lead to the activation of proliferative and survival signaling pathways, such as the Ras/Raf/MEK (mitogen-activated protein kinase, or MAPK) and Akt/mTOR cascades[15].

Abnormal expression or regulation of epidermal growth factors (EGF) and the receptors are implicated in the pathogenesis of many malignancies[16]. EGFR is overexpressed or up-regulated in colorectal cancers and pancreatic cancers, and is associated with early progression

and poor survival[17-22]. Similarly, EGFR is overexpressed in HCC and is associated with aggressive features with increased cellular proliferation and reduced apoptosis. *In vitro* inhibition of EGFR in HCC cell lines results in cell cycle arrest and apoptosis[23-25]. These led to the clinical development of anti-EGFR agents as single agent, or in combination therapy in view of their *in vitro* and *in vivo* synergistic activity with cytotoxic agents[26].

Cetuximab

Cetuximab is a chimeric murine/human IgG1 monoclonal antibody that blocks ligand-dependant EGFR receptor activation. The antibody has a higher affinity for the receptor than the ligands, such as EGF and transformaing growth factor (TGF-α)[27-29]. The drug is cytostatic when administered alone but highly synergistic with irinotecan in refractory colorectal cancer xenografts, leading to clinical development in irinotecan-refractory colorectal cancer patients[30,31]. In the pivotal multi-center randomized phase Ⅲ trial, 329 patients with metastatic colorectal cancer who progressed on irinotecan-based therapy were randomized to receive cetuximab alone or a combination of cetuximab and irinotecan[9]. The patients in the combination arm achieved a superior response rate of 22.9% and median time to progression of 4.1 mo compared to 10.8% and 1.5 mo in the monotherapy arm respectively. The median survival was not statistically different between the two groups.

Compared to best supportive care, metastatic colorectal cancer patients who failed multiple previous regimens achieved better overall survival, time to progression and quality of life with cetuximab monotherapy in the recent study by National Cancer Institute of Canada Clinical Trials Group (NCIC-CTG) and Australasian Gastro-Intestinal Trials Group (AGITG)[32]. In the first line setting, Cetuximab improved response rate and time to progression when administered in combination with irinotecan-based regimen (FOLFIRI) in the CRYSTAL trial[33].

The efficacy of cetuximab with oxaliplatin-based regimen (such as FOLFOX) in second- and first-line settings is being evaluated in randomized trials (the EXPLORE and OPUS trials, respectively)[34-36]. However, the addition of cetuximab to oxaliplatin based fluoropyrimidine regimens (FOLFOX or CapOx) seemed to increase the frequency of grade 3/4 adverse events, specifically gastrointestinal toxicities, rash and lethargy[37]. The role of cetuximab in adjuvant, or postoperative, setting is being studied in 2 ongoing randomized trials (PETACC-8, Intergroup 0147) in combination with oxaliplatin-containing regimens[38-40].

Cetuximab is approved by FDA in U.S. for use in patients with EGFR-expressing colorectal cancer who failed previous irinotecan-based therapy. This was due to the fact that the trials mentioned enrolled only patients with EGFR-expressing tumors, based on preclinical data suggesting the predictive value of EGFR expression for cetuximab efficacy. However, patients with EGFR-negative colorectal cancer were later found to benefit from cetuximab therapy as well, suggesting that EGFR expression level does not correlate with cetuximab response[41,42]. This is an important lesson for the development of biological agents: patient selection based on expression, or non-expression, of specific molecular markers can be faulty. Such hypothesis should be validated vigorously in well-designed clinical trials.

The side effects of cetuximab are fairly tolerable with appropriate management. Hypersensitive infusion reaction was reported in about 3% of the patients. About 75% of patients receiving cetuximab developed a mild acneiform-like rash. The development of cetuximab-related rash seemed to correlate with response but this needs to be studied further[43].

Cetuximab was evaluated in combination with gemcitabine in advanced pancreratic cancer. Despite encouraging phase Ⅱ results, the recent randomized phase Ⅲ trial (SWOG S0205) failed to confirm the superiority of cetuximab plus gemcitabine combination over gemcitabine monotherapy in this patient population[44].

Cetuximab monotherapy proved to be tolerable in patients with advanced HCC though activity was lacking in phase Ⅱ trials[27,45]. Gruenwald *et al* enrolled 32 unresectable HCC patients and 27 were evaluable. Seventy-two percent (23 of 32) had Child-Pugh Stage A cirrhosis, 25% Stage B and 3% Stage C. Previously treated patients were eligible for this trial and 44% achieved stable disease for at least 8 wk and median time to progression was 22.5 wk. The agent is been evaluated in combination with cytotoxic chemotherapy in HCC[46].

Panitumumab

Panitumumab is a fully humanized anti-EGFR monoclonal antibody that is being evaluated in metastatic colorectal cancer. The agent has the advantage of avoiding the hypersensitive reaction typical of chimeric murine proteins, such as cetuximab. In a multi-institutional phase Ⅲ trial, patients with refractory metastatic colorectal cancer were randomized to receive panitumumab plus best supportive care or best supportive care alone[47]. Eight percent (8%) of patients receiving panitumumab achieved partial response. About 90% developed the characteristic acneiform rash comparable to cetuximab monotherapy. As expected and importantly, hypersensitivity infusion reaction for the humanized monoclonal antibody was lower than that reported for cetuximab. Combination regimens containing panitunumab are been evaluated clinically.

Erlotinib

Erlotinib is an oral quinazoline that reversibly inhibits EGF receptor tyrosine kinase. The small molecule induces in vitro cell cycle arrest and apoptosis, and has *in vivo* anti-tumor effects[48,49]. Major side effects are rash and diarrhea, characteristic of this class of drug. Erlotinib was approved in 2004 by FDA in U.S. for use as single agent in previously treated non-small cell lung cancer (NSCLC) following the demonstration of survival benefit in a randomized phase Ⅲ trial (NCIC-CTG BR.21)[50]. EGFR mutations seems to correlate with the efficacy of anti-EGFR therapy in NSCLC though effort to uncover additional molecular predictors continues[51].

Among GI cancers, erlotinib is furthest along clinical development in pancreatic cancer. Gemcitabine has been

Table 1 Agents targeting EGFR pathway in GI cancers

Agents	Tumor types	Regimen	Study design	References
Monoclonal antibodies				
Cetuximab	Colorectal cancer	Irinotecan/cetuximab	Phase Ⅲ	[9]
	Hepatocellular carcinoma	Cetuximab	Phase Ⅱ	[27]
	Pancreatic cancer	Gemcitabine/cetuximab	Phase Ⅱ	[28]
	Pancreatic cancer	Gemcitabine/RT/cetuximab	Phase Ⅱ	[29]
Panitumumab	Colorectal carcinoma	Panitumumab	Phase Ⅲ	[47]
Matuzumab	Pancreatic cancer	Gemcitabine/matuzumab	Phase Ⅰ	[60]
	Colorectal cancer	Matuzumab	Phase Ⅰ	[61]
Tyrosine kinase inhibitors				
Erlotinib	Pancreatic cancer	Gemcitabine/erlotinib	Phase Ⅲ	[52]
	Colorectal cancer	CapOx/erlotinib	Phase Ⅱ	[54]
	Hepatocellular carcinoma	Erlotinib	Phase Ⅱ	[56]
	Colorectal cancer	FOLFIRI/erlotinib	Phase Ⅰ	[55]
	Pancreatic cancer	Gemcitabine/paclitaxol/RT/erlotinib	Phase Ⅰ	[62]
Geftinib	Colorectal cancer	Gefitinib/fluorouracil/oxaliplatin	Phase Ⅱ	[63]
	Colorectal cancer	Gefitinib/oxaliplatin	Phase Ⅱ	[64]
	Colorectal cancer	Gefitinib	Phase Ⅱ	[65,66]
	Hepatocellular carcinoma	Gefitinib	Phase Ⅱ	[67]
	Pancreatic and rectal cancer	Capecitabine/gefitinib/RT	Phase Ⅰ	[68]
Lapatinib	Colorectal cancer	Lapatinib	Phase Ⅱ	[59]

RT: Radiation therapy.

the standard first-line therapy for advanced pancreatic cancer in improving symptoms and survival, but not curative[6]. In the NCIC-CTG sponsored multi-institutional trial, 569 patients with untreated advanced pancreatic adenocarcinoma were randomized to receive gemcitabine plus erlotinib or gemcitabine plus placebo[52]. Intension-to-treat analysis showed longer survival in patients receiving erlotinib plus gemcitabine (6.24 mo *vs* 5.91 mo; HR 0.82, $P = 0.038$) compared to gemcitabine only. One year survival was also higher in the erlotinib-containing arm (23% *vs* 17%, $P = 0.023$). Unlike colorectal cancer, tumor EGFR expression was not a pre-requisite in this trial. There was more frequent mild grade rash, diarrhea and hematological toxicity in the combination arm but the frequency of moderate and severe toxicities were comparable in both arms. However, routine use of erlotinib and gemcitabine combination cannot be recommended in patients with advanced pancreatic cancer in view of the high cost of erlotinib[53].

Erlotinib use in colorectal cancer remains investigational. The drug showed encouraging result when used in combination with capecitabine and oxaliplatin in previously treated disease in phase Ⅱ trial[54]. The result needs to be validated in a larger randomized trial. The drug had unacceptably high rate of toxicity when combined with dose-reduced FOLFIRI in patients with metastatic colorectal cancer[55].

Erlotinib is being tested in untreated advanced HCC patients in an ongoing open-labeled phase Ⅱ trial[56]. Tumor EGFR expression is not an exclusion criteria in this trial. Interim analysis of 25 patients suggested a longer median survival among erlotinib-responding patients of 44 wk compared to 25 wk in erlotinib-non-responders. All responders developed rashes. The trial aims to accrue a total of 40 patients.

Lapatinib

Lapatinib is an interesting oral inhibitor of two tyrosine kinases: ErbB1 (EGFR) and ErbB2 (HER-2/*neu*). The agent has significant efficacy in advanced breast cancer when combined with capecitabine[57]. Both EGFR and HER-2/*neu* are co-expressed in colorectal cancer cells and simultaneous targeting of these receptors in preclinical studies enhanced apoptosis. Lapatinib is currently being tested in previously treated colorectal cancer patients[58,59].

EGFR pathway proves to be a valid target in GI cancers, especially in colorectal cancer with cetuximab and panitumumab. The small but statistically significant survival improvement by erlotinib in pancreatic cancer has been more a demonstration of "proof-in-principle" and the optimal approach to using anti-EGFR agents in pancreatic cancer still needs to be defined. Lapatinib development will hopefully shed light on whether dual-targeting of the ErbB receptor family is a successful approach in colorectal cancer (Table 1).

ANGIOGENESIS

Angiogenesis is vital to cellular growth, reproduction and development[69]. The process is often pathological in cancers, driven by an imbalance of pro- and anti-angiogenic factors in tumors[70]. The resulting tumor-induced vasculature is often leaky and dysfunctional, leading to increase interstitial pressure that impedes the delivery of both oxygen and chemotherapeutic agents[71].

VEGF-A (commonly known as VEGF) is among the first angiogenic factor discovered and shares sequence homology to the platelet-derived growth factor (PDGF) superfamily[72,73]. VEGF-A interacts with two transmembrane receptor tyrosine kinases: VEGFR-1 (Flt-1) and VEGFR-2 (KDE, Flk-1). VEGFR-2 is the primary mediator of VEGF-A and is often overexpressed in tumor vasculatures. Activation of VEGFR-2 promotes endothelial cell proliferation, survival and migration. As such, VEGFR-2 has been a major anti-angiogenic target.

VEGF over-expression and increased microvessel

density correlated with disease recurrence, metastases and survival in colorectal cancers[74-84]. Similarly, increased VEGF expression in pancreatic adenocarcinoma was also associated with poor prognosis though some studies suggest that PDGF and bFGF, instead of VEGF-A, are more important in the modulation of angiogenesis in pancreatic cancer[85-88]. HCC is highly vascular and patients with the liver neoplasm have higher serum VEGF levels than those with benign liver tumors[89-91]. In addition, increased VEGF expression following surgical resection or prior to transarterial chemoembolization correlated with poor prognosis[92-95].

As such, angiogenesis has been a focus of GI cancer therapy and can be accomplished by monoclonal antibody and small molecule tyrosine kinase inhibitor. These anti-angiogenic agents are believed to exert their anti-tumor effects by either affecting the tumor directly, inhibiting neovascularization, or enhancing chemotherapy delivery by normalizing the tumor vasculature[71,96].

Bevacizumab

Bevacizumab is a humanized monoclonal VEGF-binding antibody with anti-angiogenic properties that is the furthest along clinical development in its class. The drug was approved by FDA in U.S. for use with intravenous fluorouracil-containing regimens in patients with metastatic colorectal cancer[97].

The hint for bevacizumab efficacy in colorectal cancer in first-line setting was observed in a phase Ⅱ trial. 104 patients with metastatic colorectal cancer were randomized to receive fluorouracil and leucovorin (5FU/LV) (control arm), 5FU/LV plus "low dose" bevacizumab (5 mg/kg) and 5FU/LV plus "high dose" bevacizumab (10 mg/kg)[98]. Patients in both bevacizumab-containing arms achieved higher response rate (control: 17%; "low dose" bevacizumab: 40%; "high dose": 24%), longer time to progression and median survival (13.8 mo; 21.5 mo; 16.1 mo, respectively). Interestingly, outcome was better in the "low dose" bevacizumab arm than the "high dose" arm and was attributed partly to a higher proportion of poor risk patients in the "high dose" arm. Bevacizumab-related toxicities in this trial included thrombosis, hypertension, proteinuria and epistaxis. Bevacizumab at 5 mg/kg was thus chosen as the recommended dose for further development.

Bevacizumab was subsequently tested in metastatic colorectal cancer patients in combination with 5FU, leucovorin, leucovorin and irinotecan (IFL) in the pivotal phase Ⅲ trial. 813 patients with untreated metastatic colorectal cancer were randomized to receive IFL plus placebo (control arm), IFL plus bevacizumab 5 mg/kg or 5FU/LV plus bevacizumab 5 mg/kg[10]. IFL superseded 5FU/LV as the standard first-line regimen in U.S. by the time this trial was planned and was chosen as the control arm. The 5FU/LV plus bevacizumab arm was added as a backup since the safety of IFL plus bevacizumab was unknown. The 5FU/LV/bevacizumab arm was discontinued later during the planned interim analysis when IFL plus bevacizumab proved to be safe. The superior survival of 20.3 mo in the IFL plus bevacizumab over the IFL plus placebo arm of 15.6 mo supported the use of bevacizumab in the first-line treatment of metastatic colorectal cancer. Consistent with the earlier phase Ⅱ trial, reversible hypertension and proteinuria were more frequent with bevacizumab use. Other rare but serious side effects include gastrointestinal perforation, thrombosis and wound dehiscence.

Bevacizumab was also tested in metastatic colorectal cancer combined with oxaliplatin-based regimen in second-line setting. In the randomized phase Ⅲ trial (E3200), patients with previously treated colorectal cancer were randomized to 3 arms: FOLFOX4 plus bevacizumab, FOLFOX4 and bevacizumab only. The dose of bevacizumab chosen was 10 mg/kg[99]. The patients were not exposed to bevacizumab previously. Preliminary result showed superior survival and progression free survival in the FOLFOX4 plus bevacizumab arm. In a separate analysis, 56% of patients receiving FOLFOX4 plus bevacizumab had bevacizumab dose reduction but the survival was not significantly different from those without dose reduction[100]. Preliminary results indicate that bevacizumab is equally effective with oxaliplatin-based regimen and should be considered in second-line setting for metastatic colorectal cancer patients without previous bevacizumab exposure.

Despite the progress with bevacizumab in metastatic colorectal cancer therapy, many clinical questions remained unanswered, such as the role of continuing bevacizumab from first- into second-line setting and the synergism of bevacizumab with oral fluoropyrimidines. The combination of bevacizumab, erlotinib plus FOLFOX was examined in a phase Ⅱ trial but 40% of patients developed unacceptable toxicity and the treatment was stopped[101]. Bevacizumab is been tested with FOLFIRI in an ongoing phase Ⅱ trial involving patients with metastatic colorectal cancer[102].

The combination of bevacizumab and gemcitabine was been evaluated in pancreatic cancer. The multi-center phase Ⅱ trial demonstrated a modest partial response rate of 21% in untreated advanced pancreatic cancer patients treated with the combination[103]. Unfortunately, the combination failed to achieve survival improvement compared to gemcitabine only therapy in the subsequent phase Ⅲ randomized trial (CALGB 80303)[104]. The combination of bevacizumab with gemcitabine plus oxaliplatin (GemOx) is being evaluated in an ongoing North Central Cancer Treatment Group phase Ⅱ trial[105].

VEGF-Trap

VEGF-Trap (Regeneron) is a novel chimeric decoy receptor with higher affinity for VEGF-A than monoclonal antibodies[106]. The molecule consists of the extracellular domains of VEGFR-1 and -2 fused to the constant region (Fc) of IgG1[107]. Preclinical studies demonstrated potent anti-tumor and anti-angiogenic activities in various cancer models, prompting further clinical testing of the agent[108,109]. Phase I study of the agent in patients with advanced solid tumors showed that the agent is well-tolerated and the toxicities, including fatigue, pain, constipation and arthralgia, can be managed safely[110]. VEGF-Trap is being tested with fluorouracil-based regimens in phase I trials[111,112].

Table 2 Agents targeting angiogenesis in GI cancers

Agents	Tumor types	Regimen	Study Design	References
Monoclonal antibodies				
Bevacizumab	Colorectal cancer	Bevacizumab/IFL	Phase Ⅲ	[10]
		Bevacizumab/FOLFOX (E3200)	Phase Ⅲ	[99]
		Bevacizumab/FOLFIRI	Phase Ⅱ	[102]
	Pancreatic cancer	Bevacizumab/gemcitabine	Phase Ⅱ/Ⅲ	[103]
		Bevacizumab/gemcitabine/oxaliplatin	Phase Ⅱ	[105]
		Bevacizumab/capecitabine/RT	Phase Ⅰ	[124]
VEGF decoy				
VEGF-Trap	Solid tumors	I-LV5FU2/ VEGF-Trap	Phase Ⅰ	[111]
	Solid tumors	FOLFOX4/ VEGF-Trap	Phase Ⅰ	[112]
Tyrosine kinase inhibitors				
Sorafenib	Hepatocellular carcinoma	Sorafenib	Phase Ⅲ	[8]
	Pancreatic cancer	Gencitabine/sorafenib	Phase Ⅰ	[116]
	Colorectal cancer	Oxaliplatin/sorafenib	Phase Ⅰ	[115]
Sunitinib	Colorectal cancer	Irinotecan/cetuximab/sunitinib	Phase Ⅰ/Ⅱ	[122]
	Hepatocellular carcinoma	Sunitinib	Phase Ⅰ/Ⅱ	[121,123]

IFL: Irinotecan/leucovorin/bolus fluorouracil; FOLFOX: Oxaliplatin/leucovorin/infusional fluorouracil; FOLFIRI: Irinotecan/leucovorin/infusional fluorouracil; RT: Radiation therapy.

Sorafenib

Sorafenib (BAY43-9006) is an oral bi-aryl urea initially developed as a potent inhibitor of Raf protein[113]. The agent is also a multi-target kinase inhibitor and has significant activity against VEGFR-1, VEGFR-2, VEGFR-3 and PDGFR. As such, sorafenib is also been evaluated for its anti-angiogenic properties. The drug significantly inhibits neovascularization in colon, breast and non-small cell lung cancer xenografts in preclinical studies, marked by decreased tumor microvessel density.

Phase I trial involving patients with refractory solid tumors showed that sorafenib is fairly well tolerated. The main toxicities were diarrhea, skin rash and fatigue[114]. Downstream ERK protein was significantly inhibited at sorafenib $\geqslant$ 200 mg bid dose, indicating Raf inhibition. Partial response was observed in one (of 6) patients with HCC (400 mg bid dose) and stable disease for more than 6 mo in 6 (of 26) of colorectal cancer patients[115,116].

Sorafenib became the first agent to achieve significant survival benefit in advanced HCC in a multi-center randomized trial (SHAPR trial)[8]. 602 patients with previously untreated advanced disease with Child-Pugh Stage A cirrhosis and good performance status (ECOG PS 0-2) were randomized to receive sorafenib or placebo. Compared to the placebo arm, patients receiving sorafenib had a longer median survival (10.7 mo *vs* 7.9 mo; HR 0.69, $P < 0.01$) and time to progression (HR 0.58, $P < 0.01$). Serious side effects were similar in both groups though diarrhea and hand-foot syndrome were more frequent in those receiving sorafenib. Criticisms of the study include the generalisability of the result since majority of the patients enrolled were European and had minimal liver dysfunction. The benefit in Child's B and C patients remains unclear. Moreover, the therapy is quite costly and is a significant financial burden for most HCC patients who live in poorer developing countries. Sorafenib continues to be evaluated in HCC in combination therapy.

Sunitinib

Sunitinib (SU11248) is an oral inhibitor of VEGFR-2, PDGFR, c-kit and FLT-3. Preclinical studies showed anti-tumor activity in various malignancies, including leukemia, breast and lung cancer models[117-119]. In a phase I study, the recommended dose for sunitinib was determined to be 50 mg/d on a "4-wk-on/2-wk-off" schedule[120]. The toxicities include hypertension, thrombocytopenia, neutropenia, diarrhea, hair and skin changes. Sunitinib is being tested in HCC and in combination with irinotecan and cetuximab in previously treated metastatic colorectal cancer[121-123].

Of the anti-angiogenic agents discussed, bevacizumab proved to be an exceptionally efficacious agent in colorectal cancer when combined with conventional cytotoxic agents. However, this monoclonal antibody failed to achieve the clinical benefit expected in pancreatic cancer in combination therapy. More excitingly, sorafenib becomes the first chemotherapeutic agent to achieve significant clinical benefit in HCC (Table 2).

AKT/mTOR PATHWAY

The mammalian target of rapamycin (mTOR) is a cytosolic serine/threonine kinase that plays a central role in cell proliferation and survival[125]. The kinase is downstream to the phosphatidylinositol 3'-kinase (PI3K)/Akt signaling pathway. Activated mTOR interacts with downstream effectors, such as 4E-BP1 and p70s6K, to modulate various growth and survival-related cellular functions. The pathway is sensitive to extracellular growth factors (EGF, VEGF and IGF) and nutrients (amino-acids, glucose and oxygen).

In a series of 101 resected primary hepatoma (with 73 HCC), 15% had overexpression of phospho-mTOR and 5% had increased total mTOR protein expression[126]. In pancreatic cancers, more than 90% of the tumors contain an activating upstream ras mutation and about half of the surgically resected pancreatic cancer specimens had mTOR activation[127-131].

Loss of the suppressive PTEN gene expression, PI3K gene mutations and amplification of Akt result in constitutive activation of the upstream PI3K/Akt pathway

observed in some tumors[126-129,132-135]. Such activation increases the tumors' susceptibility to mTOR inhibitors and provided the rationale in developing rapamycin (mTOR inhibitor) analogs in various cancer types[136-140]. In addition, inhibition of mTOR reversed gemcitabine resistance in gemcitabine-resistant pancreatic cancer cell lines in preclinical xenograft model[131]. These preclinical data support the clinical testing of mTOR inhibitors in HCC and pancreatic cancer.

Rapamycin

Rapamycin (sirolimus) is an oral macrolide derived from *Streptomyces hygroscopicus* that is widely used as immunosuppressant in organ transplantation[141-145]. Rapamycin and its analogs also inhibit cellular proliferation in a wide range of human tumors. The drug complexes with FKBP12, a member of the immunophilin family of FK506-binding proteins, intracellularly which in turn inhibits the mTOR kinase activity, leading to G1 phase cell cycle arrest and apoptosis[146,147]. However, the drugs poor aqueous solubility, chemical stability and lack of investor interest impeded its clinical development as an anti-neoplastic agent[12]. Currently, rapamycin is being tested in a pharmacodynamic-guided dose-finding study involving patients with advanced solid tumor and also in a phase Ⅱ trial involving patients with advanced pancreatic cancer[148].

Temsirolimus

Temsirolimus (CCI-779) is a water-soluble synthetic rapamycin ester with significant anti-proliferative properties that can be administered *via* both oral and intravenous routes[149-154]. The drug demonstrated comparable *in vitro* anti-tumor effect to rapamycin against a wide range of human cancer cell lines, including prostate, breast, small-cell lung carcinoma, melanoma, glioblastoma and T-cell leukemia. The agent inhibits tumor growth, or is cytostatic, in a variety of cancer xenograft models but did not achieve tumor shrinkage.

Two dosing schedules of temsirolimus were tested in separate phase I trials: weekly intravenous dose versus the 30 minute intravenous infusion administered daily for 5 d on a bi-weekly schedule[155,156]. Toxicities observed include skin changes, muscostomatitis, asthenia, myelosuppression (thrombocytopenia, neutropenia), dyslipidemia and elevated liver enzymes. Dose escalation for the weekly regimen was stopped at 220 mg/m^2, which was the highest planned dose. Toxicities were fairly manageable and reversible at this dose. Interestingly, tumor shrinkages (partial and minor responses) were observed clinically, contrary to the cytostatic phenomenon seen in preclinical studies. Two patients achieved partial response: one with renal cell carcinoma and another with breast cancer. This led to further testing of temsirolimus in various cancer types[157-160]. Temsirolimus was recently approved by FDA in U.S. for the treatment of poor risk renal cell carcinoma patients based on the positive result from a randomized phase Ⅲ trial[161].

Everolimus

Everolimus (RAD001) is an oral rapamycin analog that inhibits tumor growth and angiogenesis in a dose-dependant manner and has anti-proliferative activity against a wide range of human cancers[162,163]. The optimal biologically active dose of everolimus was studied in two phase I trials. Everolimus 20 mg weekly was determined to be biologically active and toxicities associated with weekly everolimus administration were well tolerated and included anorexia, fatigue, rash, mucositis, headache, hyperlipidemia and gastrointestinal disturbance. The dose-limiting toxicities of daily everolimus were stomatitis, neutropenia and hyperglycemia. Pre-treatment and during-treatment tumor biopsies were done to evaluate pharmodynamic effects of everolimus and a 10 mg daily dose was recommended as the optimal dose. Partial response was seen in one colorectal cancer patient and everolimus is in phase Ⅱ development as single agent in refractory colorectal cancer[164]. The agent is being developed in other cancer types as well, such as gastrointestinal stromal tumor, neuroendocrine tumors, renal cell carcinoma, non-small cell lung cancer and melanoma[165-169].

The Akt/mTOR pathway seems to be an important survival and pro-growth pathway in GI cancers. Temsirolimus is the first of its class to achieve significant anti-tumor efficacy and clinical development of the class of mTOR inhibitors in pancreatic cancer and HCC continues.

CONCLUSION

Angiogenesis and EGFR pathways were hypothesized as targets for anticancer therapy more than three decades ago. Efforts to translate this knowledge to bedside are just starting to benefit patients with GI cancers. Successful development of cetuximab and bevacizumab in colorectal cancer ushered in the era of biologically targeted agents in the fight against GI cancers. More milestones were later achieved when the survival of previously difficult-to-treat GI cancers were improved by these novel biological agents, as in the case of erlotinib in pancreatic cancer and sorafenib in HCC. More molecular targets will become apparent as our knowledge of the complex neoplastic processes increases, and will provide exciting translational opportunities in the development of GI cancer therapy.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

New approaches in angiogenic targeting for colorectal cancer

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Abstract

Colorectal carcinoma (CRC) is one of the leading causes of cancer death worldwide. In the last decade, the addition of irinotecan and oxaliplatin to standard fluorouracil-based chemotherapy regimens have set the new benchmark of survival for patients with metastatic CRC at approximately 20 mo. Despite these advances in the management of CRC, there is a strong medical need for more effective and well-tolerated therapies. The dependence of tumor growth and metastasis on blood vessels makes angiogenesis a rational target for therapy. One of the major pathways involved in this process is the vascular endothelial growth factor (VEGF) and its receptors (VEGFR). In 2004, the first agent targeting angiogenesis, bevacizumab (BV), was approved as an adjunct to first-line cytotoxic treatment of metastatic CRC. The role of BV as part of adjuvant treatment and in combination with other targeted therapies is the subject of ongoing trials. However, BV is associated with an increase in the risk of arterial thromboembolic events, hypertension and gastrointestinal perforations and its use must be cautious. Novel VEGFR TK inhibitors with different ranges of nanomolar potencies, selectivities, and pharmacokinetic properties are entering phase Ⅲ trials for the treatment of cancer. Conversely, one of these novel agents, vatalanib, has been shown not to confer survival benefit in first and second-line treatment of advanced CRC. The basis of these findings is being extensively evaluated. Ongoing and new well-designed trials will define the optimal clinical application of the actual antiangiogenic agents, and, on the other hand, intensive efforts in basic research will identify new agents with different antiangiogenic approaches for the treatment of CRC. In this review we discuss and highlight current and future approaches in angiogenic targeting for CRC.

INTRODUCTION

Colorectal carcinoma (CRC) is one of the leading causes of cancer death worldwide despite progressive improvements in preventive, diagnostic, and therapeutic approaches[1]. Approximately 50 percent of patients who undergo potentially curative surgery alone ultimately relapse and die of metastatic disease[2]. From the late 50 s, 5-fluorouracil (5-FU) was the only drug approved for the treatment of advanced CRC with an overall response rate (RR) and median survival of 10% and 10 mo, respectively[3,4]. This RR was improved to nearly 25% when leucovorin (LV) was used to modulate 5-FU[5]. Recently, irinotecan and oxaliplatin have been added to the armamentarium of agents with activity in CRC. The addition of these two cytotoxic agents to the standard 5-FU/LV-based regimens improves not only RR, but also overall survival (OS) over 5-FU/LV alone, setting the new benchmark of survival for patients with unresectable advanced CRC at around 20 mo[6-10]. Despite these advances in the management of CRC, there is a strong medical need for more effective and well-tolerated therapies and further improvements in survival are anticipated with the introduction of novel targeted therapies both as single agents and in combination. Among them, anti-angiogenesis agents have become a new therapeutic approach in the metastatic setting. In this review we will discuss and highlight current and future approaches in angiogenic targeting for CRC.

ANGIOGENIC TARGETING

The dependence of tumor growth and metastasis on blood vessels makes angiogenesis one of the fundamental hallmarks of cancer[11] and a rational target for[12]. Several growth factor receptor pathways have been implicated in the promotion of tumor angiogenesis. One of the major pathways involved in this process is the vascular endothelial

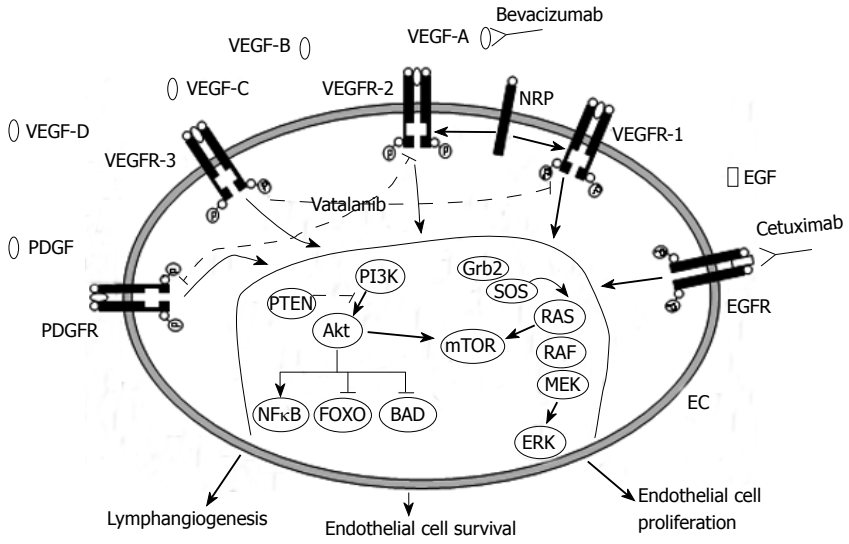

Figure 1 Vascular endothelial growth factor (VEGF) signaling network and novel targeted therapies. VEGFR: Vascular endothelial growth factor receptor; PDGF: Plateled-derived growth factor; PDGFR: Plateled-derived growth factor receptor; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; NRP: Neuropilin; EC: Endothelial cell.

growth factor (VEGF) family of proteins, also known as vascular permeability factors, and its receptors (Figure 1). The VEGF pathway plays a crucial role in normal and pathologic angiogenesis, triggering multiple signaling networks that result in endothelial cell survival, migration, mitogenesis, differentiation, and vascular permeability[13]. The VEGF-related gene family of angiogenic and lymphangiogenic growth factors comprises six secreted glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF) 1 and 2. The primary effects of VEGF ligands are mediated through binding to the VEGF tyrosine kinase receptors (VEGFR): VEGFR-1, which binds VEGF-A, VEGF-B, and PlGF-1; VEGFR-2, which binds VEGF-A, VEGF-C, VEGF-D, and VEGF-E; and VEGFR-3, which binds VEGF-C and VEGF-D, and its expression is limited to the lymphatic endothelial cells. In addition to these receptors, VEGF interacts with neuropilins, a family of activating coreceptors without an intracellular signaling domain[14,15]. VEGFR-1 and VEGFR-2 have seven extracellular immunoglobulin-like domains, a single transmembrane region and a consensus kinase sequence that is interrupted by a kinase-insert domain[16]. Once bound by VEGF, two receptors dimerize, and the tyrosine kinase domain of each receptor "autophosphorylates" the other, leading to an active receptor that initiates a signaling cascade. The VEGF pathway is upregulated by hypoxia[17] and by several growth factors, such as epidermal growth factor (EGF)[18], platelet-derived growth factors (PDGFs)[19,20], hepatocyte growth factor[21] and other cytokines.

Overexpression of VEGF has been associated with tumor progression and poor prognosis in several tumor systems, including CRC[22,23]. Preoperative serum VEGF have also been shown to correlate with advanced tumor stage or nodal status at the time of surgery[24]. Furthermore, intense expression of VEGF mRNA is detected in human liver metastases from primary colon or rectal carcinomas[25]. In 1993, Kim *et al*[26] reported that antibodies to VEGF exert a potent inhibitory effect on the growth of several tumor cell lines in nude mice. In addition, the combination of anti-VEGF antibody and chemotherapy in nude mice injected with human cancer xenografts has an increased antitumor effect compared with antibody or chemotherapy treatment alone[27]. It is, therefore, not surprising that most of the antiangiogenesis treatment strategies focus on inhibition of the VEGF pathway and its regulators. However, the mechanisms of action of anti-VEGF therapy in cancer patients are still far from being fully understood.

In December 2004 the first agent targeting angiogenesis, bevacizumab (Avastin®; Genentech, Inc., South San Francisco, CA), was approved to be given intravenously as a combination treatment along with standard chemotherapy drugs for metastatic CRC, increasing RR, progression-free survival (PFS) and overall survival (OS) with limited toxicity[28]. Gradually, many other antiangiogenic agents that target the VEGF pathway are entering the clinic. These novel targeted agents inhibit the VEGF pathway by targeting the VEGF ligand, its receptors or by blocking downstream signaling pathway components. Antiangiogenic agents include antibodies, low-molecular-weight tyrosine kinase (TK) inhibitors, antisense oligonucleotides and aptamers (Table 1).

BEVACIZUMAB IN CRC

Bevacizumab (BV) is a recombinant humanized monoclonal antibody that binds to all isoforms of VEGF-A with a reported half-life of 17-21 d[29]. In phase I trials, BV was generally well tolerated and did not demonstrate dose-limiting toxicity or interactions with commonly used chemotherapy regimens[30,31]. Based on the data obtained in these phase I trials, Kabbinavar *et al*[32] conducted a randomized, phase II trial comparing the safety and efficacy of BV (at two dose levels, 5 and 10 mg/kg every 2 wk) plus 5-FU (500 mg/m^2)/LV (500 mg/m^2) versus 5-FU/LV alone as first-line therapy for metastatic CRC(Table 2). One hundred and two patients were included. Administration of BV at low-dose and high-dose every 2 wk resulted in a significant increase of 3.8 mo and 2.0 mo, respectively, in the estimated progression-free survival (PFS) compared with 5-FU/LV alone. Treatment with 5-FU/LV/BV at both dose levels compared with 5-FU/LV resulted in

Table 1 Anti-VEGF agents currently in clinical development

Agent	Targets	Phase of development
Specific anti-VEGF antibodies		
Bevacizumab (Avastin)	VEGF-A	Phase Ⅲ
IMC-C1121b	VEGFR-2	Phase Ⅰ-Ⅱ
VEGF Trap	VEGF, PlGF, VEGF-B	Phase Ⅰ
Agents that target VEGF receptors tyrosine kinase		
Vatalanib (PTK787/ZK 222584)	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Phase Ⅲ
Sorafenib (BAY 43-9006)	VEGFR-2, PDGFR-β, FLT3, c-Kit, Raf	Phase Ⅲ
Sunitinib (SU11248)	VEGFR2, PDGFR-β, FLT3, c-Kit	Phase Ⅲ
Semaxanib (SU5416)	VEGFR1, VEGFR2	Stopped
AZD2171	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
CEP-7055	VEGFR1, VEGFR2, VEGFR3	Phase Ⅰ-Ⅱ
CHIR258	VEGFR1, VEGFR2, FGFR1, FGFR3	
CP-547632	VEGFR2	Phase Ⅰ-Ⅱ
GW786034	VEGFR2	Phase Ⅰ-Ⅱ
OSI-930	VEGFR, c-Kit	Phase Ⅰ-Ⅱ
ZK-CDK	VEGFRs, PDGFR, CDKs	Phase Ⅰ-Ⅱ
AG013736	VEGFR, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
AMG706	VEGFR1, VEGFR2, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
KRN-951	VEGFR1, VEGFR2, VEGFR3, PDGFR-β, c-Kit	Phase Ⅰ-Ⅱ
BMS-582664	VEGFR2, FGFR	Phase Ⅰ-Ⅱ
XL999	FGFR, VEGFRs, PDGFR, FLT3	Phase Ⅰ-Ⅱ
Zactima (ZD6474)	VEGFR2, EGFR, RET	Phase Ⅰ-Ⅱ
AEE788	VEGFR1, VEGFR2, EGFR	Phase Ⅰ-Ⅱ
Antisense oligonucleotides		
Veglin (VEGF-AS)	VEGF, VEGF-C, VEGF-D	Phase Ⅰ
Aptamer		
Aplidin (Dehydrodidemnin B)	VEGF	Phase Ⅰ

CDK: Cyclin-dependent kinase; EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; FLT3: Fms-related tyrosine kinase 3; MMP: Matrix metalloproteinase; PDGFR: Platelet-derived growth factor receptor; PIGF: Placental growth factor; VEGF: Vascular endothelial growth factor.

Table 2 Completed trials for Bevacizumab with chemotherapy in metastatic CRC

REF	Regimen	Pts	RR (%)	*P*	PFS or TTP (mo)	*P*	OS (mo)	*P*
28	IFL	411	35	0.004	6.2	< 0.001	15.6	< 0.001
	IFL + BV	402	45		10.6		20.3	
32	5-FU/LV	35	17	-	5.2	-	13.6	-
	5-FU/LV + BV-low	35	40	0.029	9	0.005	21.5	0.137
	5-FU/LV + BV-high	32	24	0.434	7.2	0.217	16.1	0.582
41	5-FU/LV	105	15	0.055	5.5	0.0002	12.9	0.16
	5-FU/LV + BV	104	26		9.2		16.6	
43	FOLFOX	289	9	< 0.001	4.8	< 0.001	10.7	0.0018
	FOLFOX + BV	290	22		7.2		12.5	
44	FOLFOX/bFOL/XELOX	147	22-43	NR	6.1-8.7	NR	18.2	NR
	FOLFOX/bFOL/XELOX + BV	213	41-53		8.3-10.3		24.4	
45	FOLFOX/XELOX	701	49	0.99	8.5	< 0.001	-	-
	FOLFOX/XELOX + BV	699	47		11		-	

CRC: Colorectal carcinoma; 5-FU/LV: 5-fluorouracil/leucovorin; IFL: Irinotecan/5-FU/leucovorin; FOLFOX-4: Oxaliplatin/5-FU/leucovorin; BV: Bevacizumab; Pts: Patients enrolled; REF: reference; RR: Response rate; PFS: Progression-free survival; TTP: Time to tumor progression; OS: Overall survival; NR: Not reported.

higher RR [control arm, 17%, (95% CI, 7% to 34%); low-dose arm, 40%, (95% CI, 24% to 58%); high-dose arm, 24%, (95% CI, 12% to 43%)]. Although median survival was 7.7 and 2.3 mo higher in the 5-mg/kg arm and 10-mg/kg arm, respectively, it was not statistically significant. These findings contrast with the effective higher dose administered in other tumors like non-small cell lung cancer (15 mg/kg every three weeks)[33], breast cancer (10 mg/kg every two weeks)[34] and renal cancer (10 mg/kg every two weeks)[35]. Nevertheless, the majority of subsequent CRC studies administered a BV dose of 5 mg/kg. Potential safety concerns observed in this phase Ⅱ study were thrombosis, hypertension, proteinuria, and epistaxis.

In 2004, a large (813 patients) phase Ⅲ, double-blind, randomized trial in patients with untreated metastatic CRC demonstrated that the addition of BV to IFL (irinotecan/5-FU/LV) chemotherapy prolonged OS by 4.7 mo compared with IFL alone (20.3 *vs* 15.6 mo; HR = 0.66, $P < 0.001$)[28]. The one-year survival rate was 74.3% in the group given IFL plus BV and 63.4% in the group given IFL plus placebo ($P < 0.001$). All secondary efficacy end points were also improved with the addition of BV to the chemotherapeutic regimen: PFS increased from 6.2 to

Table 3 Trials for Vatalanib with chemotherapy in metastatic CRC

REF	Regimen	Pts	RR (%)	*P*	PFS or TTP (mo)	*P*	OS (mo)	*P*
58	FOLFOX-4	583	46		7.6			
	FOLFOX-4 + Vatalanib	585	42	NS	7.7	0.118	NR	-
59-60	FOLFOX-4	429	18	NR	4.1	0.026	11.8	0.511
	FOLFOX-4 + Vatalanib	426	19		5.5		12.1	

CRC: Colorectal carcinoma; REF: Reference; Pts: Patients enrolled; FOLFOX-4: Oxaliplatin/5-FU/leucovorin; RR: Response rate; PFS: Progression-free survival; TTP: Time to tumor progression; OS: Overall survival; NS: Statistically nonsignificant; NR: Not reported.

10.6 mo (hazard ratio HR = 0.54; $P < 0.001$), RR increased from 34.8% to 44.8% ($P = 0.004$), and median duration of the response increased from 7.1 to 10.4 mo (HR = 0.62; $P = 0.001$). Grade 3 hypertension was more common during treatment with IFL plus BV than with IFL plus placebo (11.0 percent *vs* 2.3 percent, $P < 0.01$) but it was easily managed with medical treatment. Although the overall incidence of grade 3 or 4 adverse events was higher among patients receiving the combined treatment, the study did not identify hemorrhage, thromboembolism, and proteinuria as possible BV-associated adverse events. Uncommon but serious side-effects of BV included the appearance of gastrointestinal perforations (1.5%), in some instances with fatal outcome[28].

Toxicity derived from antiangiogenic therapy is a main concern in the management of CRC. BV is associated with a two-fold increase in the risk of arterial thromboembolic events, from 2.5% to 5% ($P < 0.01$)[36]. These events consist primarily of acute coronary syndrome, transient ischemic attack and stroke. Patients at risk for these events are those with a prior history of arterial thromboembolism and age older than 65 years. Moreover, BV administration can result in the development of wound dehiscence. However, the risk of wound healing is not increased if the administration of BV with or without chemotherapy is delayed until 28-60 d after primary care surgery[37].

Although the addition of BV to 5-FU-based combination chemotherapy resulted in statistically significant and clinically meaningful improvement in RR, PFS and OS among patients with metastatic CRC, previous studies have suggested that the benefit observed with irinotecan-based schedules might be limited to patients with a performance status (PS) of 0[38]; and certain subgroups, including those with advanced age, impaired PS, low serum albumin, and prior pelvic radiotherapy, may experience significant toxicities when adding irinotecan to 5-5-FU/LV regimens[39]. In this particular population, the combination of BV and 5-FU/LV would remain a potentially useful therapeutic alternative. Two studies led by Kabbinavar *et al*[40,41] addressed this question enrolling patients who were not candidates for irinotecan because of advanced age or poor PS. The results suggested that 5-FU/LV (Roswell Park Schedule[42]) plus BV seems as effective as IFL and might have a better safety profile. Based on all of the previous data, BV became the first anti-VEGF agent to be approved by the FDA for cancer patients.

On June 2006, the FDA granted approval for a labelling extension for BV in combination with intravenous 5-FU-based chemotherapy for the second-line treatment of metastatic CRC. This decision was based on the preliminary results of the E3200 phase Ⅲ trial of the Eastern Cooperative Oncology Group (ECOG). The aim of this randomized, three-arm, multicenter study was to determine the efficacy of infusional 5-FU/LV/oxaliplatin (FOLFOX) with or without BV (10 mg/kg every two weeks) in 829 patients with irinotecan-refractory advanced CRC not previously treated with BV[43]. The median age was 61 years, 49% had an ECOG performance status of 0, and 80% received prior adjuvant chemotherapy. The combination therapy showed an improvement in the OS by 2.1 mo (12.5 *vs* 10.7 mo; $P = 0.0024$) without a significant difference in the toxicity profile. The BV-alone arm was closed at the interim analysis due to a low RR and an apparent lack of activity in this setting. Final analyses of this trial are forthcoming.

Whether the combination of BV with oxaliplatin/5-FU/LV-based chemotherapy regimens will be the best option for first-line therapy for CRC is under investigation in the TREE study[44] and NO16966[45]. The TREE study was previously designed to assess the safety, tolerability and efficacy of each of three oxaliplatin plus fluoropyrimidine regimens without (TREE1 cohort) or with (TREE2 cohort) BV. In the TREE-2 cohort, BV was added to each regimen. With a follow-up of 27 mo, median OS with infusional 5-FU/LV and oxaliplatin (mFOLFOX-6) plus BV was 26.0 mo, 20.7 mo with bolus 5-FU/LV and oxaliplatin (bFOL) plus BV, and 27.0 mo with capecitabine and oxaliplatin (CapeOX) plus BV. Median OS with oxaliplatin-containing regimens without BV in sequential historical cohorts (TREE-1 study), reached 18.2 mo[44]. However, the first large, randomized, multicenter phase Ⅲ trial to evaluate the efficacy of BV in combination with the standard chemotherapy regimen FOLFOX and the XELOX regimen in the first-line treatment of metastatic CRC is the NO16966[45]. Interestingly, in the general treated population, the addition of BV to FOLFOX did not significantly improve PFS (HR = 0.89, $P = 0.1871$). However, 50% of patients discontinued treatment for reasons unrelated to progression of disease. Further analyses focusing on the on-treatment subgroup population revealed that median PFS for XELOX-BV and FOLFOX-BV was 10.4 mo compared to 8.1 mo for XELOX-Placebo and FOLFOX-Placebo (HR = 0.63, $P < 0.0001$). These results demonstrated that the addition of BV to oxaliplatin-based chemotherapy regimens significantly improves PFS. In addition, continuation of BV until disease progression could be necessary to

optimize the contribution of BV to PFS[45].

The activity shown by BV in the metastatic setting justified the evaluation of this antibody in the adjuvant scenario. In the first trial, the National Surgical Adjuvant Breast and Bowel Project C-08 phase Ⅲ trial[46], 2632 patients with stage Ⅱ or Ⅲ colorectal cancer have been randomized to receive mFOLFOX-6 for 12 cycles with or without BV. Patients assigned to BV plus chemotherapy also received an additional 6 mo of BV alone. This trial has already completed accrual. In a second trial recently finished, the AVANT phase Ⅲ study[47], patients with stage Ⅱ or Ⅲ colorectal cancer were randomized to three combination chemotherapy regimens (FOLFOX-4 *vs* FOLFOX-4 plus BV *vs* capecitabine/oxaliplatin plus BV). In addition, a phase Ⅱ clinical trial, the Eastern Cooperative Oncology Group (ECOG) E5202[48], is evaluating the addition of BV in combination with FOLFOX on patients with stage Ⅱ colon cancer at high-risk for recurrence. In conclusion, at this point in time, no evidence supports the actual use of BV in the adjuvant setting in order to prolong survival. The results of these important clinical trials are eagerly awaited.

VATALINIB IN CRC

A second antiangiogenic approach is to target both cancer cells and endothelial cells with small molecules. Similar to BV, VEGFR multitargeted TK inhibitors have been evaluated in combination with chemotherapy in phase Ⅲ trials. The first agent, semaxinib (SU5416, Pharmacia, San Francisco, California) which targets VEGFR-1, VEGFR-2 VEGFR-3, and PDGFR-β did not show any survival benefit when added intravenously to standard chemotherapy in metastatic CRC. In addition worse toxicity in the semaxinib arm was observed[49]. Finally, in a phase I trial that evaluated the combination of semaxinib with cisplatin/gemcitabine in solid tumors, an unexpected high incidence of thromboembolic events was observed which discouraged overall further investigation of this agent[50].

Another novel synthetic agent, with orally bioavailability, vatalanib (PTK787/ZK222584, Novartis, Basel, Switzerland) belongs to the chemical class of aminophthalazines[51]. It is a potent inhibitor of all known VEGFR tyrosine kinases (TK) with greater potency against VEGFR-1 and VEGFR-2[52,53] (Figure 1). It also inhibits other kinases, such as platelet-derived growth factor receptor beta (PDGFR-β) and c-Kit tyrosine kinase. In preclinical studies, vatalanib has shown antitumor activity in subcutaneously implanted human tumor xenografts in nude mice[53]. Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and pharmacokinetic (PK) data indicated that vatalanib ⩾ 1000 mg total daily dose is the biologically active dose[54] with a terminal half-life of about 6 h. In view of the short half-life of the drug, a phase I study with vatalanib given twice daily was conducted to exploit the theoretical advantage of maintaining constant drug levels[55]. PK data from this study showed that at equivalent daily doses, drug exposure is comparable with the previous once-daily-dosing schedule[54]; however, the trough levels are significantly higher with the bid dosing. Whether this will translate into improved efficacy is unknown at this time.

Vatalanib has been evaluated in two phase Ⅰ / Ⅱ studies as a single daily-dose in combination with FOLFOX or FOLFIRI, as first-line treatment for patients with metastatic CRC[56,57]. In both studies, vatalanib was safe and well tolerated at doses of 1250 mg/d. Ataxia, expressive dysphasia and dizziness were seen at higher doses when administered in combination with FOLFOX and these were considered dose-limiting toxicities. The combination of vatalanib with chemotherapy significantly affected the PK parameters of SN38, the active metabolite of irinotecan. Indeed, the concentration-time curve (AUC) of SN38 was decreased when vatalanib was added to the FOLFIRI regimen. The relevance of this finding needs further investigation.

Two phase Ⅲ studies have evaluated the administration of vatalanib (single daily-dose of 1250 mg/d) in combination with chemotherapy in CRC (Table 3). A first randomized phase Ⅲ trial (CONFIRM-1) compared the efficacy of vatalanib in combination with FOLFOX versus FOLFOX alone in 1168 patients for first-line treatment of metastatic CRC[58]. The results of the primary endpoint of this trial, PFS, showed a modest benefit of adding vatalanib to FOLFOX without achieving statistical significance (HR = 0.88; P = 0.118). OS has not been reported. The adverse events attributable to vatalanib (hypertension, deep-vein thrombosis, diarrhea and dizziness) were generally reversible and similar to other VEGF pathway inhibitors. No increase in bleeding or bowel perforation compared to placebo was observed. The second phase Ⅲ trial (CONFIRM-2) evaluated the efficacy of vatalanib in combination with FOLFOX *versus* FOLFOX alone in 855 patients with irinotecan-refractory advanced CRC[59,60]. PFS was 1.4 mo significantly longer in the vatalanib arm (5.5 mo *vs* 4.1 mo, HR = 0.83; P = 0.026). No improvement in OS was demonstrated. In the CONFIRM-2 trial, the most frequent grade 3/4 events associated with vatalanib were again hypertension (21% *vs* 5%), diarrhea (16% *vs* 8%), fatigue (14.5% *vs* 6.9%), nausea (11% *vs* 5%), vomiting (9% *vs* 5%) and dizziness (9% *vs* 1%). Two hypotheses have been tried to explain why survival was not affected when adding vatalanib in first and second-line therapy. The first one deals with the short half-life of vatalanib. The once-daily administration of the drug might not be the optimal schedule to maintain constant blood levels of vatalanib, although another study refutes this hypothesis[54]. A second one would be the "off-target" effects, such as targeting PDGFR-β. The inhibition of PDGFR-β could interfere with vascular normalization by blocking perivascular cell recruitment and thus impeding the delivery of chemotherapeutics to chemoresponsive tumors[61].

Major *et al*[62] reported a metanalysis by pooling preplanned strata in CONFIRM-1 (C1) and CONFIRM-2 (C2) trials and showed that patients with high LDH (> 1.5 X ULN) experienced the greatest improvement in PFS for C1 (HR = 0.67; P = 0.01) and for C2 (HR = 0.63; P < 0.001). This finding brings forward an eventual role of LDH in angiogenesis-dependent tumor growth and progression in CRC. Previously, the expression of LDH-5, a LDH isoform, has been linked with distant metastases in

CRC and with the expression of hypoxia inducible factor (HIF)[63]. Furthermore, evidence of a biologic link between tumor LDH, hypoxia and activated VEGF pathway has been described in CRC[64]. LDH, being regulated by the same pathway as VEGF, is expected to reflect a subset of tumors with a high likelihood to bear an activated VEGF signalling pathway. Nevertheless, whether LDH can be used as a surrogate marker for screening patients for TK inhibitor therapy remains an open question. Thus, validation of biomarkers of efficacy of anti-VEGF therapy with the aim of identifying responsive patients and predict the optimal biological dose are imperative.

TARGETED THERAPY COMBINATIONS

Growth factors and their receptors play a pivotal role in the regulation of cancer progression and neovascularization[65], stimulating downstream signaling cascades involved in cell proliferation, survival and antiapoptosis. The expression or activation of epidermal growth factor receptor (EGFR) and ErbB2 are altered in many epithelial tumors, and clinical studies indicate that they have an important role in tumor progression[66]. Inhibiting signaling pathways through EGFR and ErbB2 has become a cornerstone in the treatment of a subgroup of patients with non-small cell lung cancer and breast cancer, respectively. In CRC, cetuximab (IMC C225, Erbitux, ImClone, New York, NY), a monoclonal antibody targeting EGFR[67], has been shown to induce apoptosis of CRC cells[68], and cetuximab in combination with irinotecan (in irinotecan-refractory and EGFR expressing metastatic CRC) was found to reverse resistance to irinotecan, producing a 22.9% RR (BOND-1 Trial)[69,70]. These findings have led to the approval of cetuximab for irinotecan-refractory advanced CRC in the Unites States and, more recently, in Europe.

As it is known, the expression of proangiogenic molecules by tumor cells can be stimulated by EGFR receptor signaling[71]. Furthermore, several studies have shown that EGFR inhibitors reduce VEGF and microvessel density in tumors that regress upon EGFR blockade[72,73]. These results provide a strong rationale for combinations of anti-EGFR agents with angiogenesis inhibitors in CRC.

The safety and efficacy of concurrent administration of BV and cetuximab has been evaluated in a randomized phase Ⅱ trial in patients with irinotecan-refractory metastatic CRC (BOND-2 trial)[74]. Seventy-five patients were assigned to receive either irinotecan/cetuximab/BV (5 mg/kg every other week) or cetuximab/BV. This study presents a similar design to BOND-1 trial with BV included in both arms. The combination of cetuximab/BV, alone or with irinotecan, is tolerable, and RR and median TTP seen with the addition of BV to either arm appear favorable compared to historical controls of the BOND-1 trial. The results of the BOND-2 trial validate the design of the planned intergroup trial CALGB/SWOG 80405[75], which plans to randomize 2289 patients to receive standard chemotherapy with the addition of cetuximab, BV, or both monoclonal antibodies in first-line metastatic CRC. The primary end-point of this trial will be to detect differences in overall median survival.

SMALL-MOLECULE TK INHIBITORS IN CRC

Finally, novel VEGFR and/or PDGFR TK inhibitors with different ranges of nanomolar potencies, selectivities, and pharmacokinetic properties are entering phase Ⅰ/Ⅱ trials for the treatment of cancer[76-78]. In addition, there are now available a series of TK inhibitors that block both the EGFR and the downstream signalling molecules on the one hand and the VEGF receptor TK on the other (Table 1). Zactima (ZD6474, AstraZeneca Pharmaceuticals, Cheshire, UK) is an orally bioavailable, anilinoquinazoline derivative, multitargeted tyrosine kinase inhibitor that targets VEGFR-2, EGFR, and RET tyrosine kinases, and is currently in phase Ⅰ/Ⅱ evaluation for the treatment of cancer[79,80]. Another broad spectrum multitargeted agent, AEE788 (Novartis, Basel, Switzerland), is an oral small-molecule inhibitor of both EGFR and VEGFR tyrosine kinases[81,82]. In preclinical studies, this agent has shown growth and metastases inhibition of human colon carcinoma in an orthotopic nude mouse model[83]. Sorafenib (BAY 43-9006; Nexavar®, Bayer Aktiengesellschaft, Leverkusen-Bayerwerk, Germany, and Onyx Pharmaceuticals Inc., Emeryville, CA) targets VEGFR2 and VEGFR3, PDGFR-β, c-Kit and FLT3 (fms-related tyrosine kinase 3) and the downstream signalling molecule of EGFR known as Raf[84]. This agent efficiently inhibits both tumor-cell proliferation and angiogenesis in preclinical models, and monotherapy treatment has shown efficacy in a phase Ⅲ trial in patients with cytokine-refractory advanced renal carcinoma, which led in 2005 to the approval by the FDA for this indication[85]. In contrast with BV, the monotherapy efficacy demonstrated by Sorafenib could mimic the synergistic effect of the combination of an anti-VEGF antibody and chemotherapy[86]. The activity of Sorafenib and similar agents in the treatment of CRC needs further development. In addition, whether it will be better to target the EGFR and VEGF receptor with two compounds, each targeting one system, or to use these new class of oral duals or broad-spectrum inhibitors, is not known at this time[87].

SUMMARY AND CONCLUDING REMARKS

The increased knowledge of the VEGF signaling network and its implication in the development and progression of CRC, together with the initial positive clinical results observed with anti-VEGF therapies, makes angiogenic targeting an appropriate cancer treatment strategy. Based on the results of the completed phase Ⅲ trials, BV can increase survival when combined with standard chemotherapy in first and second-line therapy of advanced CRC. These findings have led to the approval of BV for the treatment of metastatic CRC. Simultaneously, the activity of BV in combination with 5-FU/LV-based chemotherapy regimens is being evaluated in early disease, a period when angiogenesis might be particularly critical. Results of these trials are eagerly awaited. The initial positive results of anti-VEGF therapy are not accomplished without added toxicity. Side effects of anti-VEGF agents are usually moderate compared with other

therapies, but the etiology is poorly understood. Major safety concerns have been raised by increased morbidity, and a number of treatment-related deaths from bowel perforations and cardiovascular events. Modest elevations in blood pressure occur occasionally and are easily managed with standard antihypertensive medications.

Since multiple growth-controlling pathways may be altered in cancer cells, combination antibody strategies are being explored in advanced CRC. BV is being assessed in combination with cetuximab in irinotecan-refractory metastatic CRC, based on the positive results of anti-EGFR therapies in this context. Preliminary data for this combination shows remarkable results without substantial differences about toxicity. New clinical trials with both targeted strategies in first-line metastatic CRC are recruiting patients. Combination of BV with novel VEGFR and broad-spectrum TK inhibitors also needs to be assessed in the treatment of CRC. One of these VEGFR TK inhibitors, vatalanib, combined with standard chemotherapy has been shown not to improve survival in first and second-line treatment of advanced CRC in both phase Ⅲ trials. New broad-spectrum TK inhibitors, such as Sorafenib, oppositely to the VEGF antibody, have shown promising monotherapy activity in other tumors. The basis of these findings is being extensively evaluated, and the identification of biomarkers to predict therapeutic response and optimal doses of anti-VEGF therapy is urgently needed in order to identify patients who will benefit from antiangiogenic therapy.

Angiogenesis research moves in two directions. In one hand, ongoing and new, well-designed trials will define the optimal clinical application of the actual antiangiogenic agents, and, on the other, intensive efforts in basic research will identify new agents with different antiangiogenic approaches for the treatment of CRC.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Combining chemotherapy and targeted therapies in metastatic colorectal cancer

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Abstract

Colorectal cancer remains one of the major causes of cancer death worldwide. During the past years, the development of new effective treatment options has led to a considerable improvement in the outcome of this disease. The advent of agents such as capecitabine, irinotecan, oxaliplatin, cetuximab and bevacizumab has translated into median survival times in the range of 2 years. Intense efforts have focused on identifying novel agents targeting specific growth factor receptors, critical signal transduction pathways or mediators of angiogenesis. In addition, several clinical trials have suggested that some of these molecularly targeted drugs can be safely and effectively used in combination with conventional chemotherapy. In this article we review various treatment options combining cytotoxic and targeted therapies currently available for patients with metastatic colorectal cancer.

Key words: Targeted therapy; Chemotherapy; Combinations; Clinical trials; Colorectal cancer

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INTRODUCTION

Chemotherapy remains the cornerstone of treatment of metastatic colorectal cancer (mCRC) and, with the exception of a minority of patients (pts) who are candidates for salvage surgery, the goal of chemotherapy is palliation. Remarkable and clinically relevant advances have been made in the last 5 years in the treatment of this disease, essentially owing to the introduction of combination chemotherapy regimens containing oxaliplatin and irinotecan (CPT-11)[1]. The addition of either drug to 5-fluorouracil/leucovorin (5-FU/LV) proved to significantly increase overall response rates and survival times. Indeed, median overall survival is highly correlated with the percentage of patients who receive the three cytotoxic agents in the course of their disease. Results from a Phase Ⅲ study by Falcone *et al*[2] suggested that the up-front use of a triplet combination of irinotecan, oxaliplatin and 5-FU/LV significantly improved the outcome in terms of response rate (RR) and survival times compared to a standard doublet of irinotecan and 5-FU/LV.

Interestingly, with the more recent incorporation of bevacizumab and cetuximab into the treatment armamentarium, the median overall survival (OS) has doubled from 12 mo to approximately 2 years in Phase Ⅲ trials. In fact, most recent trials that attempt to expose patients to all five drug classes (fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and anti-EGFR antibody) target an OS well over 2 years. In this review we will summarize some of the available therapeutic repertoire based on targeted therapies in combination with chemotherapy for patients with mCRC.

COMBINING CHEMOTHERAPY AND EGFR-TARGETED THERAPIES

The epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase, is one of four members of the HER receptor family. This receptor is overexpressed in a number of solid tumors of ectodermal origin, including colon adenocarcinoma[3]. EGFR over expression has been correlated with disease progression, poor prognosis and reduced sensitivity to chemotherapy[4]. Therefore, several strategies have been developed to target EGFR, including small molecule tyrosine kinase inhibitors and monoclonal antibodies[5].

Cetuximab-based combination therapy

Cetuximab is the most advanced monoclonal antibody against EGFR in clinical development. Since preclinical

and early clinical studies suggested that Cetuximab might revert irinotecan resistance in CRC both *in vitro* and *in vivo*, a phase Ⅱ trial of cetuximab with irinotecan was performed in patients with EGFR positive colorectal cancer that was refractory to both 5-fluorouracil (5-FU) and Irinotecan. Among the 120 patients treated with this regimen, overall response rate was 22.5%[6].

To confirm these clinical findings, 329 EGFR-positive, irinotecan-refractory mCRC patients were randomized in a 2:1 ratio to receive cetuximab plus irinotecan (arm A; $n = 218$) or cetuximab alone (arm B; $n = 111$) with the option to switch to the combination of cetuximab with irinotecan after failure of cetuximab as a single agent. Both the response rate (22.9% *vs* 10.8%, $P = 0.007$) and the median time to progression (4.1 *vs* 1.5, $P < 0.001$) favored the combination arm. Although no survival benefit was observed for arm A, cetuxibab was demonstrated to have clinically significant activity when given alone or in combination with irinotecan and consequently received FDA approval[7].

More recently, MABEL trial[8] investigated the combination of cetuximab and CPT-11 at a dose and schedule as pre-study in an uncontrolled, multicenter study including 1123 mCRC pts with detectable EGFR. 64% of the patients had received ⩾ 2 lines of chemotherapy. 76% had also been pretreated with cetuximab. The estimated median survival was 9.2 mo at as expense of an acceptable toxicity profile, including grade 3-4 diarrhea (20%) acne-like rash (19%), neutropenia (9%) and asthenia (8%). MABEL clearly confirmed in a wider setting the efficacy and safety or C225 plus CPT-11 seen in previous studies. Similarly, EPIC trial is a randomized phase Ⅲ trial comparing cetuximab plus irinotecan to irinotecan as second line therapy in patients with EGFR-expressing mCRC who have failed first line oxaliplatin in combination with a fluorpyrimidine. Accrual is currently ongoing[9].

Cetuximab-based combinations as salvage therapy: Several trials have addressed the potential of cetuximab-based combinations in heavily pretreated patients. Vincenzi *et al*[10] evaluated the efficacy of cetuximab plus oxaliplatin in patients previously failed on an oxaliplatin-based regimen in first line, irinotecan-based regimen in second line, and cetuximab plus irinotecan in third line. No objective clinical response was identified after the interim analysis planned according to the two-staged Simon accrual design. The same group[11] evaluated the activity of cetuximab and weekly irinotecan (90 mg/m^2) in patients refractory to one oxaliplatin-based chemotherapy regimen (Capecitabine + Oxaliplatin or FOLFOX Ⅳ regimen, as first line) and one Irinotecan-based based-chemotherapy (FOLFIRI regimen, as second-line chemotherapy) for at least 2 mo. Overall response rate was 25.4% (95% CI: 21.7%-39.6%); 38.2% (95 CI: 18.6%-39.8%) of patients showed a disease stability as the best response. The median time to progression was 4.7 mo (95% CI: 2.5-7.1 mo) and the median survival time was 9.8 mo (95% CI: 3.9-10.1 mo). The most common G3-4 noncutaneous side toxicities were diarrhoea (16.4%), fatigue (12.7%), stomatitis (7.3%) and skin toxicity (32.6%). A statistically significant ($P = 0.006$) association between the cutaneous toxicity and both tumour response and time to progression was observed. The authors also identified a borderline significant difference in terms of overall survival.

The combination of Cetuximab plus FOLFIRI has been prospectively evaluated in 41 EGFR expressing mCRC pts refractory to prior FOLFIRI for metastatic disease[12]. Most of the patients were treated in third line. A 20% overall response rate was recorded, with a median PFS of 4.3 mo and a median overall survival of 5 mo.

Cetuximab-based combinations in front-line therapy: Cetuximab established activity in the salvage setting prompted its incorporation to first-line combination therapy. Available preliminary data from Phase Ⅱ trials combining cetuximab with either irinotecan or oxaliplatin-based chemotherapy have shown very encouraging activity. CALGB 80203[13] randomized untreated mCRC patients to FOLFOX or FOLFIRI with or without C225 independent of EGFR status. ORR was similar in the FOLFIRI or FOLFOX arms, while C225 containing arms had a higher ORR (49% *vs* 33%, $P = 0.014$) when compared to non cetuximab containing arms. No significant differences in grade 3 diarrhea or any grade 4 toxicity were seen with the addition of C225. Preliminary results of the combination of C225, capecitabine (800 g/m^2 bid po on d 1 to 14) and Irinotecan (200 g/m^2 i.v on d 1) *vs* C225 combined with capecitabine (1000 mg/m^2 bid in d 1-14) and oxaliplatin (130 g/m^2 on d 1) reported an overall response rate of 41% (95%; 22% to 61%) and 71% (95%; 48% to 89%) respectively, with both arms showing a manageable toxicity profile[14].

Promising results have also been reported[15] combining cetuximab with (AIO) infusional 5-FU/FA plus irinotecan regimen in EGFR-expressing mCRC.Grade 3 or 4 toxicities were acne-like rash (38%), diarrhea (29%), cardiovascular events (20%) and nausea/vomiting (5%). Objective responses were observed in 67% of the patients. The median time to progression was 9.9 mo and the median survival time was 33 mo.

The combination of cetuximab with modified FOLFOX 6 in 83 chemo-naive mCRC pts with positive or undetectable EGFR expression show a preliminary ORR of 53%[16]. Main grade 3-4 toxicities included neutropenia (38%), diarrhea (10%), rash (10%) and neurotoxicity (7%). The combination of FOLFOX-4 plus C225[17] has also been evaluated in 47 EGFR-expressing mCRC, with a reported ORR of 68%. Grade 3-4 adverse events included acne-like rash (18%) diarrhea (7%), nausea and vomiting (4%) and anemia (4%).

Preliminary results of the OPUS trial[18], a randomized phase Ⅱ study in the first line treatment of mCRC, confirmed the superiority of FOLFOX plus cetuximab *vs* FOLFOX in terms of overall response rate (45.6% *vs* 36.8%).

These small trials supported the conduct of a multicenter Phase Ⅲ clinical trial that compared FOLFIRI plus Cetuximab with FOLFIRI alone in 1217 EGFR-expressing chemotherapy-naive patients. Cetuximab plus FOLFIRI significantly increased response rate and progression-free survival, reducing the relative risk of progression by approximately 15%[19].

Panitumumab-based combination therapy

Panitumumab is a fully human IgG2 monoclonal antibody directed against the epidermal growth factor receptor. Its use in combination with IFL and FOLFIRI in first line treatment of metastatic CRC has been evaluated in a multicenter, single arm, phase 2 trial[20]. Panitumumab was given at a weekly dose of 2.5 mg/kg i.v. over 60-90 min followed by chemotherapy. The combination with IFL was considered too toxic, with grade 3-4 diarrhea in 47% of the patients. The FOLFIRI plus panitumumab combination was associated with a more manageable side effect profile with grade 3-4 diarrhea in 25% of the pts and grade 3-4 hypomagnesemia in 8%. Skin and nail toxicities occurred in at least 20% of patients but were rarely severe (grade 3 in 2 out of 24 pts). The objective response rate with FOLFIRI plus panitumumab was 66%, with a disease control rate of 79%. Median progression free survival was 10.9 mo. Further investigation of FOLFIRI with an every two weeks schedule of panitumumab is ongoing in randomized phase 3 trials.

Cetuximab-induced papulopustular skin rash is thought to be mechanism- and dose-related, and may be a surrogate indicator of an adequate degree of receptor saturation by cetuximab. The possibility of increasing Cetuximab efficacy by inducing skin rash has been recently confirmed. Cetuximab dose escalation up to 500 mg/m^2 improves response rate in patients with absent or slight skin reaction on standard dose treatment[21].

Future directions

Large studies validating molecular predictive markers are needed in order to identify the subset of patients more likely to respond to EGFR-targeted therapies. Candidate markers include total and phosphorylated EGFR, total and phosphorylated forms of AKT, mitogen-activated protein kinase (MAPK), mitogen-activated protein/ERK (MEK), ERK, signal transducers and activators of transcription (STAT), PTEN and mTOR[22] Although EGFR gene copy number has also been proposed[23], EGFR amplification, measured by FISH is a rare event (4%) in colorectal cancer[24]. Other potential predictive markers are k-ras[25] cyclin D1 A870G polymorphisms[26], HER-2 expression[27] or higher gene expression levels of VEGF[28]. More recently, a combination of various predictive biomarkers has retrospectively been able to identify subsets of patients more likely to benefit from cetuximab therapy[29]. In addition, several polymorphisms in genes involved in the EGFR and angiogenesis pathway have been associated with clinical outcome[30]. Prospective studies are clearly needed to confirm these preliminary findings.

EGFR tyrosine kinase inhibitor (TKI)-based combination therapy

Gefitinib: Gefitinib (ZD1839) selectively inhibits the EGFR tyrosine kinase and has approximately 100-fold greater potency against EGFR compared with other tyrosine or serine/threonine kinases. Unlike cetuximab, gefitinib does not induce EGFR internalization or degradation in CRC cells, nor does it reduce EGF binding sites or EGFR protein content. Both *in vitro* and *in vivo* studies indicated that gefitinib monotherapy had antitumor activity in some CRC cell lines[31]. However, phase I / Ⅱ clinical studies in patients with mCRC indicated that gefitinib had negligible activity[32,33]. Preclinical suggestions of a supra-additive, growth-inhibitory effect of gefitinib and a wide variety of cytotoxic drugs with different mechanism(s) of action[34] prompted several trials of gefitinib in combination with chemotherapy in mCRC patients.

Gefitinib plus fluoropyrimidines: In preclinical models a strong synergistic interaction between gefitinib and 5'-deoxy-fluorouridine (5'-DFUR) was demonstrated when ZD1839 was applied before or concurrently with 5'-DFUR[35]. Subsequently, the combination of intermittent gefitinib (250-500 mg/d on d 1-14) plus 5-FU/LV administered as a bolus in a dose-reduced Mayo Clinic regimen (370/20 mg/m^2) on d 8-12 with 5-FU and leucovorin as first-line therapy in mCRC was tested, with no evidence of cumulative toxicity or major drug-drug pharmacokinetic interactions[36]. In the second part of the study, gefitinib was administered continuously at 500 mg/d, and 5-FU/LV was added to the schedule on d 8-12 and 36-40. Overall response rate was 23%, with the most common toxicities being rash and diarrhea.

Preliminary results from a small phase I / Ⅱ trial combining gefitinib 250-mg daily with capecitabine 1000-1250 mg twice daily after failure of first-line therapy[37] also suggest some evidence of activity .

Gefitinib plus irinotecan-based therapy: A dose-finding trial of irinotecan plus gefitinib in mCRC patients pretreated with fluoropyrimidine-based chemotherapy defined irinotecan given at a dose of 225 mg/m^2 every 3 wk plus gefitinib at a dose of 250 mg/d as the maximun tolerated dose (MTD) of this regimen[38]. Dose-limiting toxicities (DLTs), such as neutropenia and diarrhea, occurred at unexpectedly low doses of irinotecan. Disease stabilization was achieved in 21% of the patients.

The combination of gefitinib plus FOLFIRI in both chemotherapy-naive mCRC patients[39] and as salvage therapy[40] was considered too toxic despite reduced weekly doses of 5-FU, LV, and irinotecan.

Gefitinib plus oxaliplatin-based therapy: Gefitinib plus FOLFOX has been tested in both the first line and the salvage setting. Kuo *et al*[41] reported data on a phase Ⅱ study of one cycle of FOLFOX-4, and then additional cycles of FOLFOX-4 with 500 mg/d of gefitinib in 27 patients with documented progressive colorectal cancer after at least one chemotherapeutic regimen (usually irinotecan based). 33% of the patients achieved objective responses, whereas 48% had stable disease for a prolonged period. Response rates did not differ depending on number of prior regimens. Median event-free survival was 5.4 mo, and overall survival was 12 mo. Another feasibility study assessed the combination of gefitinib (250 mg/d) plus capecitabine (2000 mg/m^2 per day, d 1-15) plus oxaliplatin (120 mg/m^2 every 3 wk for six courses) as first-line treatment in patients with mCRC[42]. The most common grade 3 adverse events were diarrhea and neutropenia. A clinical benefit rate of 58% has been noted.

Overall, toxicity rates with the addition of gefitinib to an oxaliplatin-fluoropyrimidine combination are markedly more favorable than with the irinotecan-based regimens, although higher incidences of grade Ⅲ or Ⅳ diarrhea, nausea, and vomiting than with FOLFOX alone are noted. Further studies of TKI-based therapy for CRC are planned or recruiting.

Erlotinib: Erlotinib, an orally reversible TKI reduces intratumoral EGFR autophosphorylation[43] with no effect on EGFR expression or surface receptor density. Evidence of single agent erlotinib activity in mCRC patients derived from disease-specific phase Ⅱ studies[44] led to the design of several trials in combination with chemotherapy.

Tarceva plus fluoropyrimidines: Additive activity of capecitabine and erlotinib in tumor models[45] supported a phase 2 trial evaluating the combination of erlotinib 150 mg daily with capecitabine 1000 mg/m^2 bid. for 14 d every 3 wk in chemotherapy-naive mCRC patients. Grade 3 diarrhea (30%) grade 3 renal insufficiency (10%) and grade 3 hiperbilirrubinemia (10%) were the most troublesome toxicities. Regarding efficacy, no complete responses were achieved whereas disease control rate was 34%[46].

Tarceva plus oxaliplatin: Meyerhardt *et al*[47] reported on the results of a triplet regimen of erlotinib, 100 mg/d, capecitabine, 1650 mg/m^2 per day (d 1-14), and oxaliplatin, 130 mg/m^2 every 3 wk in 32 patients mostly pretreated with an irinotecan-containing regimen. By intent-to-treat analysis, 25% of the patients experienced a partial response and 44% had stable disease for at least 12 wk. 29% of the patients discontinued study therapy due to toxicity.

Other TKIs-based combinations

EKB-569, an irreversible dual inhibitor of the EGFR and HER-2 tyrosine kinases, inhibits the growth of tumor cells that overexpress EGFR or HER-2 *in vitro* and *in vivo*[48]. Dose-limiting toxicities with EKB-569 plus FOLFIRI in 47 chemotherapy-naive mCRC patients[49] were grade 3 diarrhea and grade 3 fatigue. The MTD was selected as 25 mg EKB-569. The response rate was 38% and the clinical benefit rate was 85%. EKB-569 treatment resulted in complete inhibition of pEGFR and significant inhibition of pMAPK in both skin samples (11 patients) and tumor samples (three patients) with no change in pAkt activity.

In a dose-escalation study[50] with FOLFOX-4 plus EKB-569, 25-75 mg/d, starting from d 3, DLTs were observed with EKB-569 at a dose of 35 mg/d (grade Ⅲ diarrhea and febrile neutropenia), leaving an MTD of 25 mg/d. The most common grade Ⅲ or Ⅳ adverse events were neutropenia (32%; 9 of 29 patients) and diarrhea (8%; 2 of 29 patients).

COMBINING CHEMOTHERAPY AND VEGF-TARGETED THERAPIES

Bevacizumab

Clinical development of Bevacizumab (BV) has rapidly progressed to Phase Ⅲ trials after a preliminary randomized Phase Ⅱ trial in which 104 previously untreated mCRC patients were randomized to two doses of BV (5 and 10 mg/kg) in addition to bolus 5-FU/LV (high dose, Rosewell-Park regimen) or to 5-FU/LV alone[51]. The combination of 5-FU/LV with low-dose BV (5 mg/kg every 2 wk) demonstrated superiority compared with the control monotherapy arm and to the BV-containing arm at a higher dose. These results provided the rationale for the key front-line Phase Ⅲ study by Hurwitz *et al*[52] which demonstrated superiority of IFL plus BV over IFL plus placebo in terms of RR (45% *vs* 35%), PFS(10.6 mo *vs* 6.2 mo) and OS(20.3 mo *vs* 15.6 mo). A subanalysis of this trial has recently stablished the benefit of Bevacizumab in mCCR patients with poor conditions[53].

The second trial (E3200) was a second-line Phase Ⅲ study, designed for patients who already failed an irinotecan-containing therapy and did not receive BV in first-line treatment[54]. Initially, the study included three randomization arms: FOLFOX4 plus BV 10 mg/kg, FOLFOX4 alone or BV 10 mg/kg alone. The BV single-agent arm was closed ahead of time since it was clearly inferior to both other arms (RR 3% and PFS 2.7 mo). The results again largely favored the BV-containing arm, especially in terms of RR (21.8% *vs* 9.2%, $P < 0.0001$) and PFS (7.2 mo *vs* 4.8 mo, $P < 0.0001$). The primary end point of the study was reached, since a statistically significant increase in median survival was obtained in the experimental arm (12.5 mo *vs* 10.7 mo, $P < 0.0024$).

Finally, updated results of N016966, a randomized phase Ⅲ trial evaluating the addition of bevacizumab to oxaliplatin-based first line chemotherapy have been reported. Bevacizumab-containing arms demonstrated a significant benefit in terms of progression-free survival, although overall response rate did not significantly differ[55].

More recently, several phase Ⅱ trials have addressed the feasibility and activity of bevacizumab when combined with various cytotoxic regimens. The First BEATrial[56] enrolled 1927 chemotherapy-naïve patients treated with a combination of bevacizumab and several first-line chemotherapies, including FOLFOX, FOLFIRI and XELOX. Median PFS was 10.4 mo. Combinations of XELOX or XELIRI plus bevacizumab have yielded tumor control rates in the range of 80% as front-line therapy for mCRC[57].

In contrast to its efficacy when used in combination with first- and second-line chemotherapy, activity of bevacizumab in chemoresistant disease has been disappointing. Chen *et al*[58] developed a treatment referral center (TRC) protocol (TRC-0301) for patients with mCRC in the third-line setting with the aim of evaluating the safety and activity of BV plus FU/LV in patients progressed after treatment with both irinotecan-based and oxaliplatin-based chemotherapy regimens. Independent review confirmed one PR (1%; 95% CI, 0% to 5.5%). Median PFS in this cohort was 3.5 mo (95% CI, 2.1 mo to 4.7 mo) and median OS was 9.0 mo (95% CI, 7.2 mo to 10.2 mo). The authors conclude that BV, alone or in combination with an ineffective chemotherapy in the third-line setting, is likely to be of minimal, if any, clinical benefit.

An important question that remains unresolved is

whether to continue bevacizumab with second-line therapy following failure of a bevacizumab-containing first-line regimen. Although retrospective data from the BRiTE trial suggest that the use of bevacizumab beyond first progression correlate with an improved survival, more mature data are required to draw any firm conclusion[59].

VEGF Tyrosine kinase inhibitors (TKI)-based combination therapy

Tyrosine kinase inhibitors of vascular endothelial growth factor receptors (VEGFRs) are low molecular weight, ATP-mimetic proteins that bind to the ATP-binding catalytic site of the tyrosine kinase domain of VEGFRs, resulting in a blockade of intracellular signaling. Several of these molecules have entered clinical evaluation.

Semaxanib: Semaxanib is a small, lipophilic, synthetic molecule that inhibits VEGFR-1, and -2 tyrosine kinases[60]. A promising response of 31.6% was observed with semaxanib at two different dose levels, 85 and 145 mg/m^2 twice weekly in combination with fluorouracil plus leucovorin as first-line therapy for 28 patients with mCRC[61]. However, a randomized, multicenter, phase Ⅲ trial failed to show any improvement in clinical outcome with semaxanib in combination with fluorouracil and leucovorin (Roswell Park regimen) *versus* fluorouracil and leucovorin alone as first-line therapy for 737 mCRC patients; moreover, worse toxicity in the semaxanib arm (in terms of diarrhea, cardiovascular events, vomiting, dehydration, and sepsis) was observed[62].

Vatalanib: Valatanib is a synthetic, low molecular weight, orally bio-available agent that inhibits all known VEGFR tyrosine kinases, platelet-derived growth factor receptor beta (PDGFR-β) and c-Kit tyrosine kinase[63].

Vatalanib was evaluated in two phase Ⅰ/Ⅱ studies as a single daily dose in combination with FOLFOX-4 or FOLFIRI. In the first study, the pharmacokinetics and toxicity profiles of both vatalanib and FOLFOX-4 were unaffected by co-administration[64]. The reported response rate was 54%, with a median PFS of 11 mo and an estimated median OS time of 16.6 mo . In the second study[65], co-administration of vatalanib at 1250 mg/d with FOLFIRI had minor effects on irinotecan exposure but lowered by 40% the AUC of SN-38 in patients' serum. The response rate was 41%, with a median PFS duration of 7.1 mo and a median OS time of 24.3 mo. Two large, randomized, double-blinded, placebo-controlled, phase Ⅲ trials compared the efficacy of oral vatalanib in combination with FOLFOX-4 with FOLFOX-4 alone in patients with mCRC, and none of them met the primary end points. In the CONFIRM-2 trial, the addition of PTK/ZK to FOLFOX-4 in previously treated mCRC did not meet the primary end points of the study. OS was 12.1 mo in the PTK/ZK arm and 11.8 mo in the placebo arm. The overall response rate was, respectively, 18.5 and 17.5%. PFS was significantly longer in the PSK/ZK arm (5.5 mo *vs* 3.8, $P = 0.026$) As in confirm 1 trial, patients with pretreatment high LDH showed a strong improvement in PFS[66]. Adverse events were similar to those of the CONFIRM-1 trial. Thrombotic and embolic events of all grades occurred in 6% of the patients treated with PTK/ZK *vs* 1% in the placebo arm. Trying to further analyze the relation between LDH levels and clinical outcome with PTK/ZK, Fixed paraffin embedded tumor samples from 36 mCRC not included in the CONFIRM trials were analyzed and tumor gene expression correlated with serum levels of LDH in the same group of patients. Intratumoral levels of LAMA, hipoxia inducible factor 1 (HIF-1), Glut-1 and VEGFA were significantly correlated. Moreover, patients with high serum LDH showed increased intratumoral gene expression of VEGFA, supporting the hypothesis of serum LDH levels as a surrogate maker for activation of the hypoxia inducible factor related genes in the tumor[66].

AZD2171: Preliminary data of a phase I evaluation of AZD2171, a highly potent and selective inhibitor of VEGFR signaling, in combination with several chemotherapy regimens including FOLFOX-6 and CPT-11, has shown some evidence of activity[67].

Vandetalib: Vandetalib, a once-daily oral inhibitor of VEGFR-dependent tumor angiogenesis, EGFR- and RET-dependent tumor proliferation, in combination with FOLFOX6[68] or FOLFIRI[69] has also shown some evidence of activity in mCRC, with diarrhea and neutropenia being the most frequent grade 3 toxicities.

Future directions

So far, clinical, biochemical, and molecular markers have failed to discriminate which patients are more likely to benefit from bevacizumab-containing regimens. An analysis of predictive markers showed indeed that bevacizumab increased the activity of irinotecan plus FU/LV regardless of the level of VEGF expression, thrombospondin expression, and microvessel density[70]. Mutations of *k-ras*, *b-raf*, and *p53* could not predict for a prolonged survival on bevacizumab plus irinotecan plus bolus FU/LV[71]. Recently, Shaye *et al* evaluated functionally significant polymorphisms of genes involved in the angiogenesis pathway in mCRC patients who receive bevacizumab as part of their front-line therapy. There were statistically significant associations between genomic polymorphisms of KDR, CXCR2, MMP7, leptin and both progression-free survival and response rate. Hopefully, prospectively collected samples from patients enrolled onto cooperative group studies and the development of selective micro arrays to define the angiogenesis-related genes in individual tumors, and at different stages of therapy and tumor progression may allow improved therapeutic efficacy.

COMBINATION OF TARGETED THERAPIES

The assumption that most advanced solid tumors derive their growth advantage from more than a signaling pathway and the significant level of compensatory cross talk among receptors within a signaling network as well as with heterologous receptor systems has provided the basis of a combined molecular targeting approach, in which more than one class of inhibitor is applied simultaneously.

A phase Ⅱ study with the combination of FOLFOX,

bevacizumab (5 mg/kg) and erlotinib (150 mg/d) every two weeks in 31 chemotherapy naive mCRC patients has been recently conducted. Grade 3-4 adverse events included diarrhea (29%) neutropenia (29%) rash (18%), fatigue (14%) and neuropathy (11%) 78% of the patients had at least one grade 3-4 toxicity. Remarkably, as much as 42% of the patients came off for toxicity. Similar results have been reported in the DREAM-OPTIMOX3 study, with a 70% incidence of grade 3-4 toxicity when adding erlotinib to a combination of bevacizumab and XELOX[73].

A phase Ⅱ trial of FOLFOX plus bevacizumab and cetuximab in 67 chemotherapy-naïve mCRC patients yielded a 55% response rate, with a median PFS of 9.6 mo and 71% of the patients progression-free for at least 8 mo[74].

The combination of FOLFOX or FOLFIRI with panitumumab and AMG706, an oral multikinase inhibitor targeting VEGF, PDGF and Kit receptors has been tested in 45 mCRC patients, with no apparent PK/PD interactions and an overall response rate in the range of 50%[75].

Based on these results, combinations of monoclonal antibodies are currently being actively tested in first-line therapy of mCRC. The Cancer and Leukemia Group B (CALGB)/South West Oncology Group (SWOG) Intergroup 80405 Phase Ⅲ trial randomizes patients to either cetuximab or bevacizumab, or both antibodies in combination, with the oncologist's choice of FOLFOX or FOLFIRI. In addition, the Panitumumab Advanced Colorectal Cancer Evaluation (PACCE) trial is currently evaluating the efficacy of FOLFOX or FOLFIRI (depending on the investigator choice) plus BV, versus the same combination plus panitumumab.

OTHER TARGETED THERAPIES-BASED COMBINATIONS

Cell cycle inhibitors

Kortmansky *et al*[76] reported the results of the combination of 5-FU and UCN-01, a selective inhibitor of a number of serine-threonine kinases, including calcium and phospholipid-dependent protein kinase C and cell cycle specific kinases, among 35 patients with advanced solid tumors, the majority of them with a diagnosis of mCRC. No objective responses were observed, although eight patients had stable disease. Most of the patients with stable disease had previously received and progressed on 5-fluorouracil. There was minimal toxicity attributed to the combination, although expected toxicities associated with UCN-01 were observed.

Apoptosis modifiers

Bcl-2 plays a pivotal role in the regulation of caspase activation and apoptosis. Its overexpression is found in 30%-94% of clinocopathological colorectal carcinoma specimens and confers a multidrug resistant phenotype in several cell lines. In support of this data, antisense oligonucleotide therapy directed against bcl-2 was shown to significantly enhance the chemosensitivity in several cancer cell lines compared with controls *in vitro*. A recently published phase I trial assessed the feasibility and pharmacokinetic behaviour of the combination of oblimersen sodium, a phosphorothioate antisense oligonucleotide that hybridizes to the first six codons of the bcl-2 open reading frame mRNA, with CPT-11 in 20 pts with mCRC. Among them, 1 pt experienced a PR while 10 additional patients had stable disease lasting 2.5-10 mo. The authors recommend oblimersen at 7 g/kg/d, d 1-8 with CPT-11 280 mg/m^2 on d 6 once every 3 wk was the RD for further development in phase Ⅱ trials[77].

Proteasome inhibitors

The proteasome inhibitor Bortezomib (PS-341), at a dose of 1.3 mg/m^2 administered twice weekly every 21 d in pretreated patients with mCRC did not prove to have clinical activity[78].

The main nonhematologic toxicities were elevation of alkaline phosphatase, constipation, fatigue, nausea, and sensory neuropathy. A phamacokinetic and phamacodynamic analysis of topotecan plus PS-341 in 22 patients with advanced solid malignancies found that, with the addition of PS-341, peripheral blood mononuclear cells (PBMC) topoisomerase I levels got stabilised or increased. These findings suggest that PS-341 may overcome resistance to topoisomerase I inhibitors, since in vitro exposure to campothecin results in down-regulation of the target enzyme. Preliminary data of the combination of FOLFOX4 plus bortezomib in mCRC patients[79] show evidence of clinical activity, with bortezomib at a dose of 1 mg/m^2 being the RD for phase Ⅱ trials.

COX inhibitors

Numerous clinical trials are ongoing to test the efficacy of nonsteroidal anti-inflammatory COX-2 inhibitors in combination regimens for therapy of advanced solid tumors[80]. Preliminary data on the combination of rofecoxib (50 mg/d) with weekly irinotecan and infusional fluorouracil demonstrated a good tolerability up to the irinotecan dose of 125 mg/m^2/wk. The phase Ⅱ study showed a 36.7% objective response rate, a clinical benefit of 76.7% and a median TTP and overall survival of 4 and 9 mo, respectively. The combination was feasible and safe, with a reduced rate of mucositis and diarrhea[81].

However, in the BICC-C trial[82], addition of celecoxib to several Irinotecan/fluorpyrimidine combinations did not impact safety or efficacy. Results of larger studies seem warranted.

Histone deacetylase inhibitors

Histone acetylation by histone acetyltransferases is important for promoting the action of several transcription factors. Acetylation facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of the target genes[83].

Vorinostat, a novel histone deacetylase inhibitor that potentiates 5-FU through a decrease in thymidylate synthase (TS) expression has been tested in combination with FOLFOX, in a phase I study that enrolled mCRC patients who had failed prior FOLFOX, irinotecan and

cetuximab therapy. Tolerance was acceptable, and some evidence of both, clinical activity (SD in some patients) and biological activity (down regulation of TS) are suggested[84].

mTOR inhibitors

Rapamycin displays potent antimicrobial and immunosuppressant effects as well as antitumor properties. Rapamycin's antiproliferative actions are due to it's ability to modulate key signal transduction pathways that link mitogenic stimuli to the synthesis of proteins necessary for the cell cycle to progress from the G1 to S phase[85].

Rapamycin clinical development has been hampered due to the poor aqueous solubility and chemical stability of the macrolide. CCI-779, a rapamycin ester derived from 2, 2-bis (hydroxymethyl) propionic acid, is one analog that was selected for further development due to its promising pharmacological, toxicological and antitumor profiles[86].

A phase I study of escalating doses of CCI-779 in combination with 5-FU/leucovorin in patients with advanced solid tumors, including mCRC reported preliminary evidence of activity including 1 complete response in a patient with mCRC receiving the 15 mg/m^2 dose and several patients with stable disease of a maximum duration of 12 mo. Further studies are required to determine appropriate regimens with this combination treatment[87].

CONCLUSION

In conclusion, the biological agents have clearly increased the therapeutic armamentarium of patients with metastatic CRC and offer also prospects for an increased chance of a longer survival. Eventually, the availability of more predictive biological factors may allow oncologists to tailor individualized targeted combination therapy to a specific patient with a specific tumor. However, the cost of novel therapies for mCRC is particularly high. Such a heavy economical burden may be counterbalanced either by a very significant breakthrough in treatment efficacy or by selection of patients with a higher chance of responding to a specific treatment.

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TOPIC HIGHLIGHT

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Epidermal growth factor receptor inhibitors in colorectal cancer treatment: What's new?

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Abstract

Colorectal cancer constitutes one of the most common malignancies and the second leading cause of death from cancer in the western world representing one million new cases and half a million deaths annually worldwide. The treatment of patients with metastatic colon cancer comprises different regimens of chemotherapeutic compounds (fluoropyrimidines, irinotecan and oxaliplatin) and new targeted therapies. Interestingly, most recent trials that attempt to expose patients to all five-drug classes (fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and cetuximab) achieve an overall survival well over 2 years. In this review we will focus on the main epidermal growth factor receptor inhibitors demonstrating clinical benefit for colorectal cancer mainly cetuximab, panitumumab, erlotinib and gefitinib. We will also describe briefly the molecular steps that lie beneath them and the different clinical or molecular mechanisms that are reported for resistance and response.

Key words: Epidermal growth factor receptor inhibitors; Cetuximab; Panitumumab; Erlotinib; Gefitinib; Metastatic colorectal cancer; Tyrosine kinase inhibitors; Monoclonal antibodies

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and the second leading cause of death from cancer in Europe and North America. It is responsible for approximately one million new cases and half a million deaths per year worldwide[1].

Several options are currently available for the treatment of patients with metastatic colorectal cancer (mCRC), including different regimens of chemotherapeutic compounds (fluoropyrimidines, irinotecan and oxaliplatin) and targeted therapies such as bevacizumab and cetuximab. Interestingly, most recent trials that attempt to expose patients to all five drug classes (fluoropyrimidines, irinotecan, oxaliplatin, bevacizumab and cetuximab) target an overall survival (OS) well over 2 years.

In this review we will summarise state-of-the-art targeting of the epidermal growth factor receptor (EGFR) in the management of metastatic colorectal cancer.

BIOLOGY OF EGFR

EGFR belongs to the ErbB family[2]. This family is comprised by transmembrane proteins that form part of the tyrosine kinases receptor proteins which are activated by different kinds of ligands[3] (Figure 1). All the receptor tyrosine kinases share the same protein structure with an extracellular binding domain, a transmembrane domain and an intracellular domain where the catalytic domain is located. The autophosphorylation of tyrosine residues outside the catalytic domain stabilises the receptor in the active conformation and recruit different proteins required for signalling.

There are several ligands binding ErbB including EGF, TGF alpha, Neuregulin family and some others[4]. Not all the ligands 'fit' all the receptors and this feature also has its implications at a molecular level[2]. Once the ligand binds the receptor and the molecule is phosphorylated it can switch on several pathways including the RAS-RAF-MAPK, JAK-STAT and the PIK3-AKT pathways. The signalling pathways activated by different EGF ligands drive various transcription factors to the nucleus that result in different cellular responses such as proliferation, migration, differentiation or apoptosis.

There are four different receptors in the ErbB family named ErbB1 (EGFR; HER or c-erbB the first to be described), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). In the active conformation, the protein forms homodimers or heterodimers that are stabilised by the ligand binding. HER2/neu cannot (due to a genetic mutation) bind to EGF-like ligands and ErbB3 does not have a functional tyrosine kinase.

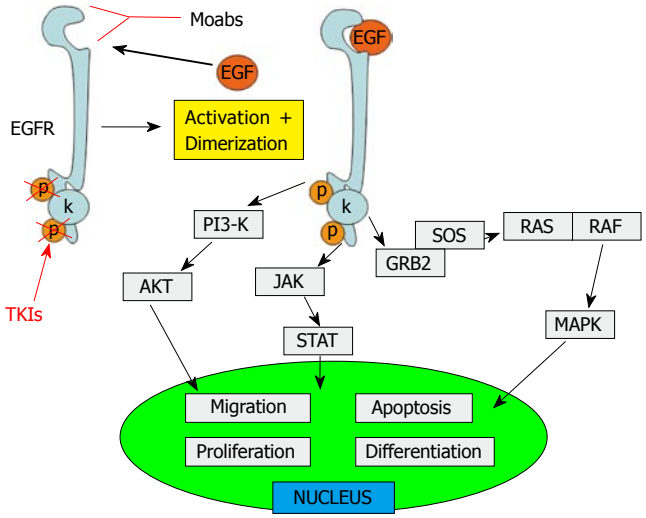

Figure 1 EGFR and its pathways.

Targeting the ErbB network may be achieved by inhibiting the tyrosine kinase (catalytic domain) with small molecules (TKIs) or by inhibiting the extracellular domain with monoclonal antibodies (Moabs) as shown in Figure 1. The moabs block the interaction between natural ligands and the EGF receptor in the extracellular space. The receptor is internalized and that can affect the network, as the timing of this process in the physiological state of the receptor also has its molecular implications[4,5]. Certain antibody isotypes such as IgG1 (cetuximab) have the potential for mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and complement fixation[6], improving thus their antitumor activity. The TKIs compete with the ATP in their binding sites on the catalytic domain of the receptor and so act inside the cell.

CLINICAL APPLICATION

Monoclonal antibodies

Cetuximab: Cetuximab is an IgG1 monoclonal antibody targeting EGFR. Since preclinical data suggested that cetuximab might revert irinotecan resistance *in vitro*[7,8] and *in vivo*[9], a phase Ⅱ study[10] with 121 EGFR expressing mCRC patients refractory to irinotecan was started. A 17% overall response rate (ORR) was documented at an expense of acceptable toxicity grade 3-4. Cetuximab monotherapy has also proved activity in irinotecan refractory patients[11]. A phase Ⅱ open-label clinical trial with 57 EGFR positive mCRC patients was treated and an ORR of 9% was observed. The acne-like skin rash was the main described toxicity related to the drug. Two patients experienced grade 3 allergic reaction and discontinued the study. The study CO.17 that compared cetuximab and best supportive care (BSC) against BSC alone showed that cetuximab provides palliation in pretreated patients with advanced CRC, delaying deterioration in quality of life as well as improving survival[12] (Table 1).

These data led to the design of a study with 329 patients (pts) refractory to irinotecan who were randomized to cetuximab (111 pts) or irinotecan plus cetuximab (CI)

Table 1 Cetuximab in Irinotecan refractory mCRC

	Pts (*n*)	RR (%)	PFS (mo)	OS (mo)
C225 + Innotecan[10]	121	17	-	-
C225[11]	57	9	-	6.4
C225 + Innotecan[13]	329	23	-	8.6

Pts: Patients; mCRC: Metastatic colorectal cancer; RR: Response rate; PFS: Progression free survival; OS: Overall survival; mo: Months; C225: Cetuximab.

(218 pts). The ORR was 22.9% (95% CI: 17.5% to 29.1%) in the CI arm as opposed to 10.8% (95% CI: 5.7% to 18.1%) in the cetuximab arm. OS (8.6 mo *vs* 6.9 mo) and time to progression (TTP) (4.1 mo *vs* 1.5 mo) also favoured the CI arm. The toxicity presented in the CI group was very similar to that of patients treated with irinotecan alone[13] (Table 1).

More mature data regarding the role of CPT-11 and cetuximab in irinotecan refractory patients have been recently reported in the MABEL trial[14]. A multicenter study with 1461 CPT-11 refractory mCRC EGFR positive patients, 64% of whom had received two or more chemotherapy lines; 1123 patients are currently evaluable and a 12-week overall progression free survival (PFS) rate is 61% (58%-64%), and 34% (31%-37%) at 24 wk. The current estimate of median survival is 9.2 mo (8.7-9.9) with grade 3/4 adverse events being diarrhea (20%), skin toxicity (including acne-like rash) (19%), neutropenia (9%) and asthenia (8%). Hypersensitivity reactions occurred in 1.5% of the patients.

The above mentioned results provided the rationale for the BOND2 study that compared the combination of irinotecan, bevacizumab and cetuximab against bevacizumab plus cetuximab in CPT-11 refractory mCRC patients. A 43% ORR as opposed to 27% in favour of the irinotecan arm was presented. The median time to progression was 7.1 mo *vs* 4.6 mo and the median survival was 18.0 mo *vs* 10.3 mo for the irinotecan group[15,16]. The toxicity observed was the expected for each agent alone.

A variety of preclinical data have suggested activity of cetuximab in oxaliplatin resistant tumors[17]. Thus, a phase Ⅱ trial that combined CAPOX (oxaliplatin 85 mg/m^2, d 1, and capecitabine 2000 mg/m^2, d 1-7, every 2 wk) plus Cetuximab in patients who had progressed to oxaliplatin-based regimens has recently been presented[18]. Eighty percent of the 40 patients had also progressed on prior irinotecan-based chemotherapy. The study achieved 1 complete response (CR) (2.5%) and 7 partial responses (PR) (17.5%) with a 20% ORR and a 47.5% disease control rate (DC). The median TTP was 3 mo and the median survival 10.7 mo. Toxicity included grade 3-4 neutropenia (12.5%) and diarrhea (7.5%) and grade 2-3 neurotoxicity (22.5%). The second trial named EPIC is a phase Ⅲ study comparing cetuximab plus irinotecan and irinotecan as a second line in EGFR positive patients who received oxaliplatin plus fluoropyrimidines as a first line therapy. The primary endpoint was overall survival and quality of life being one of the secondary endpoints. Cetuximab plus irinotecan (n = 648) was superior to irinotecan alone (n = 650)

regarding progression-free survival and response rate (16.4% *vs* 4.2%, $P < 0.0001$). OS was comparable between both arms, but it may have been influenced by crossover. Health related quality of life was better preserved on the combination arm with less deterioration in symptom scores (pain, nausea, insomnia) and better health status scores[19]. Main toxicity (> 10%) grade 3-4 were neutropenia (30%) and diarrhea (21%). There is also a study by Lenz *et al*[20] analyzing with 346 refractory to irinotecan, fluoropyrimidines or oxaliplatin EGFR positive patients that achieved a RR of 12% with cetuximab monotherapy in patients.

The preliminary promising efficacy seen with C225 in refractory mCRC has prompted its use as front line therapy. In the ACROBAT study 43 EGFR positive mCRC patients were treated with cetuximab plus FOLFOX with a 77% RR, a median survival of 30 mo and a median PFS of 12.3 mo[21]. The study presented by Rosemberg *et al*[22] in 2002 was designed as a phase Ⅱ study with 27 EGFR positive patients that were treated with irinotecan, 5-fluorouracil/leucovorin (IFL) and cetuximab as frontline. They showed a 44% PR rate with another 20% of patients showing minor responses. Twenty-six out of 27 patients presented with rash, but only 19% were grade 3. Another study with a similar chemotherapeutic scheme was presented by Folprecht *et al*[23] in 2005 with a 67% RR and 29% stable disease rate in 20% of whom their liver metastases were resected after treatment. They used high and normal doses of 5-fluorouracil/leucovorin, three out of fifteen patients presented dose limiting toxicity (DLT) in the group of high dose (2000 mg/m^2). A phase Ⅱ study with 23 EGFR positive mCRC patients of whom 22 were assessable for response were treated with FOLFIRI and cetuximab in first line therapy. It showed a 46% PR rate and a 41% SD rate with a median TTP of 10.9 mo. Most common grade 3/4 toxicities were diarrhea, neutropenia and rash[24]. Seven patients underwent secondary surgery of metastases. Another study with FOLFOX-6 plus cetuximab in chemo-naive patients showed a preliminary 53% ORR with 3 CR[25]. It was a phase Ⅱ study with 82 mCRC patients showing positive or undetectable EGFR expression. 14 patients discontinued the study due to toxicity and 10% of the patients had grade 4 neutropenia and 2% grade 4 sepsis (Table 2).

More recently, results of the CRYSTAL study, a phase Ⅲ clinical trial that compares FOLFIRI plus cetuximab (arm A) *versus* FOLFIRI alone (arm B) in 1217 mCRC have been presented. The median PFS was significantly longer for arm A compared to arm B [8.9 mo (CI: 8-9.5) for group A *versus* 8 mo (CI: 7.6-9) for group B, $P = 0.036$]. RR was also significantly increased by cetuximab (46.9% *vs* 38.7%, $P = 0.005$). The most common toxicities were neutropenia (26.7% in group A, 23.3% in group B), diarrhea (15.2% and 10.5% respectively) and skin reactions (18.7% and 0.2% respectively)[26]. The OPUS study is a phase Ⅲ clinical trial[27] that randomized patients to FOLFOX or FOLFOX plus cetuximab in chemo-naive patients. Their primary objective was response rate and secondary objectives were PFS, OS, and the R0 resection rate after metastatic surgery of curative intent. The preliminary results showed an RR of 35.7% and 45.6% respectively with 337 patients enrolled at that time. The most common grade 3/4 adverse events were neutropenia (27.6% in A; 31.5% in B), diarrhea (7.1% and 6.0%), leucopenia (7.1% and 5.4%) and rash (9.4% in the cetuximab arm only). The COIN study is a phase Ⅲ trial[28] (804 pts) comparing either continuous chemotherapy plus cetuximab or intermittent chemotherapy with the standard palliative combination. The addition of cetuximab to oxaliplatin-fluoropyrimidine combinations results in increased grade 3/4 toxicities overall and specifically to the gastrointestinal (GI), skin rash and lethargy. Capecitabine combination is associated with more GI toxicity but less neutropenia. Unexpectedly, no hypersensitivity reactions have been seen yet on FOLFOX (with or without cetuximab) (Table 3).

Table 2 Cetuximab as frontline, Phase Ⅱ studies

C225 plus:	Pts (*n*)	RR (%)	PFS (mo)	OS (mo)
FOLFIRI[25]	22	80	10.9	-
FOLFOX-4[22]	43	77	12.3	30
FOLFOX-6[26]	82	53	-	-

Pts: Patients; RR: Response rate; PFS: Progression free survival; OS: Overall survival; C225: Cetuximab.

Table 3 Cetuximab as frontline, Phase Ⅲ studies

C225 plus:	Pts (*n*)	RR (%)	PFS (mo)	OS (mo)
FOLFOX Cetuximab *vs* FOLFOX[28]	337	46.6% *vs* 35.5%	-	-
FOLFIRI Cetuximab *vs* FOLFIRI[27]	1217	46.9% *vs* 38.7%	8.9 *vs* 8.0	-

Pts: Patients; RR: Response rate; PFS: Progression free survival; OS: Overall survival.

Panitumumab: Panitumumab is a fully human IgG2 monoclonal antibody directed against the epidermal growth factor receptor. Several trials have tested its role in pretreated mCRC. The study with 148 mCRC refractory to FOLFOX/FOLFIRI EGFR positive patients treated with panitumumab alone showed a 10% RR with 36% of SD. 90% of the patients appeared with skin rash but only 4% G3[29]. Another study with panitumumab in refractory patients to FOLFOX/FOLFIRI[30] showed benefit for treating those patients with Panitumumab *vs* BSC. They were 463 EGFR positive patients who were assigned to panitumumab or BSC alone. The median progression free survival was 8 wk in the Panitumumab group *vs* 7.3 wk in the BSC group and the mean PFS 13.8 wk *vs* 8.5 wk. The RR was 10% in the Panitumumab group and 0% in the BSC group. The main toxicities were rash, diarrhea and hypomagnesemia. They did not find any advantage in overall survival due to the crossover but it resulted in a 46% reduction in the risk of tumor progression. Another study with 91 mCRC pretreated patients with negative or low EGFR by immunohistochemistry (IHC) showed a 7%-9% PR rate with 36%-42% of DC presenting skin and hypomagnesemia as main toxicities[31] (Table 4).

Table 4 Panitumumab, Phase Ⅱ and Ⅲ studies

	Pts (*n*)	RR (%)	PFS	Naive	Phase
Alone[30]	148	10	-	No	Ⅱ
Alone *vs* BSC[31]	463	10	8 wk	No	Ⅲ
Alone[32]	91	8	8 wk	No	Ⅱ
IFL + Panitumumab *vs* FOLFIRI + Panitumumab[33]	19	46	5.6 mo	Yes	Ⅱ
	24	42	10.9 mo		

Pts: Patients; RR: Response rate; PFS: Progression free survival; OS: Overall survival; mo: months; BSC: Best supportive care.

Panitumumab showed better tolerability combined with FOLFIRI than with IFL[32]. In a pooled analysis of several trials[33] the skin toxicity in panitumumab patients was 90%-95% but only in 3%-5% was grade 3 and treatment limiting. The other relevant toxicities were gastrointestinal (nausea, diarrhea and anorexia) which accounts for 25%-30% of all grades (2% grade 3) and hypomagnesemia (41%; 7% grade 3). The severity of skin rash was correlated with increased efficacy in terms of ORR, PFS, and OS[34,35]. A recent study with panitumumab has correlated skin toxicity with increased efficacy and better health-related quality of life[34]. In this phase Ⅲ study patients were randomized to panitumumab plus BSC (231 patients) or BSC alone (232 patients) and the skin toxicity was analyzed in relation to PFS and OS. The incidence of grade 2-4 skin toxicity was higher in the panitumumab arm. OS was significantly prolonged in patients with more severe skin toxicity (gr 2-4 *vs* gr 1; HR = 0.67; $P = 0.0235$) (Table 4).

Tyrosine kinase inhibitors

Gefitinib: Gefitinib is a potent, specific EGFR tyrosine kinase activity inhibitor. Phase Ⅰ/Ⅱ trials in patients with mCRC showed little activity[36,37] but preclinical studies *in vitro* and *in vivo* suggested a supra-additive growth inhibitory effect of gefitinib when combined with different cytotoxic drugs[38] which gave support to several clinical trials of gefitinib combined with chemotherapy in mCRC patients.

The study by Magné *et al*[39] support studies that combined gefitinib with fluoropirimidines[40]. The study was designed in two parts with 23 patients overall. One part with intermittent dose-escalated gefitinib plus 5-fluorouracil (370 mg/m^2 Ⅳ)/LV (20 mg/m^2 Ⅳ) and the other with continuous gefitinib at the safest dose assigned by part one. The safest dose assessed was 500 mg/d achieving a 23% OS with skin rash and diarrhea as main toxicities. Preliminary results from a small phase Ⅰ/Ⅱ trial combining gefitinib 250 mg/d plus capecitabine 1000-1250 mg bid. after failure to first line therapy also suggests some evidence of activity[41].

A dose-finding trial was performed with irinotecan plus gefitinib in 18 patients with advanced CRC refractory to fluoropyrimidine-based chemotherapy. It defined irinotecan given at a dose of 225 mg/m^2 as a single agent every 3 wk plus gefitinib at a dose of 250 mg/d as the maximum tolerated dose (MTD) of this regimen[42]. Dose-limiting toxicities, such as neutropenia and diarrhea, occurred at unexpectedly low doses of irinotecan. Disease stabilization was achieved in 21% (4 out of 18 patients). Once they achieved the recommended dose level (RDL) they expanded the study to a multicenter one with a total of 27 patients at the RDL with an objective tumor response rate of 11% and median survival 9.3 mo[43]. The toxicity grades 3-4 included diarrhea (35.9%), lethargy (15.4%), neutropenia (15.4% with 10.3% febrile neutropenia) and skin rash (7.7%).

The combination of gefitinib plus FOLFIRI in both chemo-naive mCRC patients[44] and as salvage therapy[45] was considered too toxic despite dose reduction in 5-fluorouracil, leucovorin and irinotecan. Toxicity was also the main issue when combining gefitinib with capecitabine in patients who had previously received one or two chemotherapy lines being diarrhea and neutropenia, the principal related DLTs[46].

In a study by Kuo *et al*[47] with 27 patients who had previously received at least one regimen (oxaliplatin based mainly) they employed FOLFOX-4 and gefitinib at a dose of 500 mg/d. 33% of the patients achieved objective responses and 48% showed stable disease. Median OS was 12.0 mo, while median event-free survival was 5.4 mo. For first-line treatment, a 74% RR with a clinical benefit rate of 98% and a median TTP of 9.5 mo. was reported by Zampino *et al*[48] with the FOLFOX-6 regimen plus gefitinib at a dose of 250 mg/daily.

The study by Zeuli *et al*[49] assessed the doses of gefitinib (250 mg/d) plus capecitabine (2000 mg/m^2 per day, d 1-15) and oxaliplatin (120 mg/m^2 d 1) every 3 wk for six courses as first-line treatment in patients with metastatic disease. The most common grade 3 adverse events were diarrhea and neutropenia. A 50% response rate (6 out of 12 patients; 5 PRs, 1 CR) and a clinical benefit rate of 58% (7 out of 12 patients) were communicated.

In an *in vitro* study working with cetuximab-resistant cell lines, authors observed that gefitinib or erlotinib retained the capacity to inhibit growth of tumor cells that were highly resistant to cetuximab[50]. These data suggest that tyrosine kinase inhibitors may further modulate intracellular signalling that is not fully blocked by extracellular anti-EGFR antibody treatment. A phase Ⅰ/Ⅱ study that combined cetuximab and gefitinib[51] presented 56% of PR in mCRC patients. This observation deserves further evaluation.

Erlotinib: Erlotinib is a small molecule that competes with ATP for the intracellular tyrosine kinase domain of EGFR, thereby inhibiting receptor autophosphorylation and blocking downstream signal transduction (Figure 1). Evidence of single agent erlotinib activity *in vitro* and in mCRC patients, derived from disease specific phase Ⅱ studies[52,53], led to the design of several trials in combination with chemotherapy. One phase Ⅱ study presented a PR rate of 4% in 51 mCRC patients. 46 of them were assessed for response. Skin rash was observed in 62% of the patients (13% G3) and grade 3 diarrhea and nausea were also observed after erlotinib monotherapy. Another phase Ⅱ study on 38 mCRC patients treated with 150 mg of erlotinib in a continuous daily schedule presented a 39% SD rate, as the best response, with rash and diarrhea as the main toxicity events[53]. Additive activity of erlotinib when combined with

capecitabine in preclinical studies with human xenografts[54] supported a phase Ⅱ study with 10 pts evaluating the combination of erlotinib 150 g daily with capecitabine 1000 mg/m^2 bid. for 14 d in chemotherapy-naive metastatic CRC patients. Grade 3 diarrhea (30%), grade 3 renal insufficiency (10%) and grade 3 hyperbilirubinemia (10%) were the most troublesome toxicities. Regarding efficacy, no complete responses were achieved whereas disease control rate (PR + SD) was 34%[55].

In the study by Meyenhart *et al*[56] when combining oxaliplatin, capecitabine and erlotinib patients started receiving 1000 mg/m^2 bid. of capecitabine that was reduced to 750 mg/m^2 bid for 14 d after the first 13 patients experienced excess of grade 3/4 toxicities. Thus, the final doses were capecitabine 750 mg/m^2 bid. for 14 d, oxaliplatin at 130 mg/m^2 on d 1, and erlotinib 150 mg daily. The ORR was 20%. In addition, the group of Delord *et al*[57]presented a dose-finding study establishing erlotinib 100 mg/d, capecitabine 1650 mg/m^2 qd (d 1-14), and oxaliplatin130 mg/m^2 every 3 wk as the MTD for this regimen.

Erlotinib (50-150 mg/d) is also being investigated in combination with FOLFOX-4 for untreated or minimally pretreated patients with CRC, with a preliminary reported 43% response rate. The most commonly communicated grade 3 or 4 toxicities were diarrhea and neutropenia[58].

CLINICAL AND MOLECULAR MARKERS OF RESISTANCE AND RESPONSE TO EGFR INHIBITORS

A peculiar toxic effect of cetuximab is a papulopustular skin rash, generally on the face and upper torso, which is thought to be mechanism- and dose-related[59]. Findings suggest that there is a correlation between intensity of skin rash and response and survival[13]. This correlation is particularly striking in a subgroup analysis from the IMC 0144 trial reported by Pippas *et al*. In that trial, patients with no skin toxicity presented no objective responses and had a median survival of 1.7 mo, whereas those who experienced grade 3 skin rash had a 20% RR and a median survival of almost 1 year[60]. This is the first reported observation of a clinical feature that may predict the clinical outcome of an antitumor agent. Dose-escalation schedules are currently under investigation in order to explore the possibility of increasing cetuximab efficacy by inducing skin rash.

The EVEREST study was designed as a phase Ⅲ trial with cetuximab escalated-doses. They started with standard dose and increased dose every 2 wk until skin toxicity grade 2 or 500 mg/m^2 of cetuximab were achieved. The dose-escalation of up to 500 mg/w indicated improvement of RR in pts with no or slight skin reactions on standard dose treatment[61] with 166 patients included in the study. The mechanism underlying the correlation between skin toxicity and tumour response is currently unclear, however, some research groups hypothesized that the rash is a surrogate indicator of an adequate degree of receptor saturation by cetuximab. If this is the case, targeting doses to achieve a desired level of cutaneous toxicity may further increase the efficacy of this agent. While this is an appealing prospect from a potential efficacy point of view, it would suggest, if true, that there might be a narrow therapeutic window when working with this drug[59].

In early clinical trials, EGFR positivity on tumor specimen by IHC was mandatory for the use of cetuximab. However today, EGFR expression status is known not to be a predictive factor of response to cetuximab since major responses in patients with EGFR negative tumors are expected after cetuximab treatment. In fact, responses have been reported by some authors[62] and nowadays EGFR status is not mandatory for the management of CRC patients[63]. Several factors might explain this apparent discrepancy, such as low sensitivity of IHC, cytological heterogeneity of CRC and differential EGRF expression in primary and metastatic tumor niches[64,65]. There are other reasons that might explain these striking data. Two distinct EGFRs have been identified in A431 cells by epidermal growth factor-binding studies. These are a major class of low-affinity EGFR (representing approximately 95% of the receptors) and a minor class of high-affinity EGFR (representing approximately 5% of the receptors), with binding affinities differing by an order of magnitude[66-68]. The current EGFR IHC detection systems used today derived from A431 cells do not distinguish between these two distinct EGFRs. It is known that high-affinity EGFRs are the biologically active receptors that switch the ErbB pathway whereas low-affinity receptors do not contribute significantly[66,69]. Another possible explanation is related to the ADCC capacity of cetuximab antibodies and two polymorphisms related to fragment C of the immunoglobulin G that are related to progression and survival[70].

In order to assess response to EGFR inhibitors in the clinical practice different molecular approaches are being evaluated. There are some studies where they try to find a correlation between some germinal polymorphisms involved in angiogenesis, the EGFR pathway, DNA repair and drug metabolism[15,71]. In a recent study they found a correlation, in patients treated only with cetuximab, between a Cyclin D1 polymorphism (A870G) and overall survival[72]. The Cyclin D1 is a protein related to p27^{KIP1} which is involved in the G1 phase arrest produced by EGFR inhibitors and that is correlated to apoptosis in tumor biopsies of patients treated with gefitinib[73]. The heterozygous AG genotype was significantly related to higher overall survival. Patients with AA homozygous genotype survived a median time of 2.3 mo (95% CI 2.1, 5.7) compared to those having homozygous GG genotype that survived a median of 4.4 mo (95% CI 1.8, 9.8). Even patients with a heterozygous AG genotype presented in comparison, a median survival of 8.5 mo (95% CI 5.5, 11.7), ($P < 0.05$)[72]. Another study showed similar results finding a correlation between EGFR (G497C GA), Cox-2 (G-765C CC) and EGF (A61G GG) polymorphisms and PFS[74].

Furthermore, a different investigation treated mCRC patients with cetuximab or panitumumab assessing the

EGFR copy number and the mutation profile of the EGFR catalytic domain and of selected exons in KRAS, BRAF, and PIK3CA[75] in the tumor sample. They found that in 8 out of 9 patients with an objective response the EGFR copy number was increased whereas only 1 out of 21 non-responders had an increased EGFR copy number. A retrospective study showed a linkage between EGFR mRNA levels by RT-PCR and TTP but not with survival[76] and found no correlation between any other ErbB receptors or EGFR by IHC and clinical outcome. There are other studies that suggested a correlation of KRAS mutation and poor outcome in terms of response and survival[77-79]. In the study by Finocchiaro *el al*[77] they analyzed tumor blocks from 85 colorectal cancer patients for EGFR expression (IHC and FISH), HER2 (FISH) and KRAS (mutation). EGFR FISH positive patients (41 patients) had a significantly higher RR and TTP than EGFR FISH negative individuals (44 patients). EGFR expression assessed by IHC was not associated with any clinical endpoint. Increased HER2 gene copy number predicts early escape from cetuximab therapy. Compared to patients with wild type KRAS, KRAS mutation carriers (32 patients) had a significantly lower RR (6.3% *vs* 26.5%, $P = 0.02$), shorter TTP (3.7 mo *vs* 6.3 mo, $P = 0.07$) and shorter survival (8.3 mo *vs* 10.8 mo, $P = 0.2$). In 22 patients with available primary and metastatic tumor samples, there was no difference between these sites for EGFR FISH, HER2 FISH and KRAS results. A study of 59 mCRC patients treated with cetuximab plus chemotherapy looked for KRAS mutations using first direct sequencing and two sensitive methods based on SNaPshot and PCR-ligase chain reaction (LCR) assays. They compared clinical response with gene mutations. No KRAS mutation was found in the 12 patients presenting clinical response. On the contrary KRAS mutation was associated with disease progression ($P = 0.0005$) and TTP was significantly decreased in patients with mutated KRAS tumors (3 mo *vs* 5.5 mo, $P = 0.015$)[78].

The other important mutations associated with the activity of EGFR inhibitors that are related to response to TKIs in lung cancer are mutations in exons 18, 19 and 21[80,81]. In mCRC it seems not to be the case. That may be due to the fact that those mutations are not commonly found in mCRC patients[20,82,83]. Because of this issue other predictive factors of response to Gefitinib such as the insulin receptor isoform A are currently under research[84].

FUTURE DIRECTIONS IN EGFR TARGETING

Monoclonal antibodies

EMD 72000: EMD 72000 (Matuzumab) is a humanized IgG1 anti-EGFR MoAb. It has completed phase I clinical testing in EGFR-positive solid tumors. 22 patients of different origin (including colorectal) received EMD 72000 weekly[85] and a 23% RR was demonstrated. EMD 72000 administered to 22 patients with colon (15 patients), gastric, or renal tumors demonstrated PR in 2 patients and a minor response in 1 patient[86] all of them with colon cancer. Another phase I study showed near-complete EGFR signalling suppression at the 1200 mg dose level[87]. A phase I study of matuzumab administered weekly to 26 patients (18 of which had CRC) showed 2 PR, and 10 SD in patients with colon cancer. In addition a preliminary analysis of skin biopsies showed that matuzumab produced inhibition of pEGFR and pMAPK with a decrease in Ki67 expression and an increase in p27[88].

AEE788: AEE788 is an oral inhibitor against EGFR, ErbB2, VEGFR-2 and KDR. A phase I study in these patients with advanced CRC and liver metastases showed the lack of clinical activity of AEE up to 400 mg with an inhibitory effect of 100%, 90% and 39% over pEGFR, pMAPK and Ki67 respectively by IHC in tumor biopsies[89]. Another study that investigated the effects of AEE *in vitro* and in biopsies from 22 advanced colorectal cancer patients did not find any major clinical responses even at the higher dose schedule (400 mg). Laser scanning cytometry quantitative analysis confirmed the target inhibition of AEE *in vitro* and in wound-induced skin pairs[90]. The lack of significant target inhibition in tumors has to do with the lack of clinical activity of AEE in this cohort of patients and is consistent with other studies.

HKI-272: HKI-272 is an irreversible pan-erbB receptor tyrosine kinase inhibitor. It inhibits the growth of tumor cells that express erbB-1 and erbB-2 (HER-2) in culture and in xenografts. HKI-272 also inhibits the growth of cultured cells that contain sensitizing and resistance-associated EGFR mutations[91]. A phase I study with 73 patients is ongoing and the preliminary results for 51 patients (3 of which are mCRC) showed a MTD of 320 mg/d with diarrhea as the DLT. Two breast cancer patients had confirmed partial responses and 2 had unconfirmed PRs[92].

Other MoAbs directed against EGFR have recently undergone clinical testing e.g., hR3[93] and ICR62[94].

NEW GENERATION OF TYROSINE KINASE INHIBITORS

Additional oral TKIs currently under clinical evaluation, include the reversible dual EGFR/Her-2 TKI lapatinib and the irreversible EGFR TKI EKB-569.

Lapatinib: Lapatinib is a reversible inhibitor of ErbB1/ErbB2 tyrosine kinases. 64 patients (22 with colon cancer) were included in a phase I study. One CR and 22 SD were achieved. Most of the patients with SD overexpressed either ErbB1 or ErbB2. The most frequent toxicities presented were rash, diarrhea, nausea/vomiting, fatigue, and anorexia. Serum VEGF may be a potential biomarker for lapatinib activity[95]. A study in combination with FOLFOX-4 to assess the safety included 13 patients (2 colon). The dose of lapatinib 1500 mg/d with FOLFOX-4 was well tolerated although 2 patients had grade $\geqslant$ 3 hematological toxicities, which resolved after delay of the next cycle. Seven patients were evaluable for response and 2 PR, 2 SD and 3 PD were confirmed[96]. A phase Ⅱ study with lapatinib as the single-agent in 86 mCRC patients who progressed to prior therapy showed 5 patients who experienced clinical benefit with stable disease

for ⩾ 20 wk[97]. The median TTP and overall survival were 8 and 42.9 wk respectively. The most commonly encountered adverse events were diarrhea (45% grade 1-2, 5% grade 3), rash (33% grade 1-2, 2% grade 3), fatigue (27% grade 1-2, 2% grade 3), nausea (20% grade 1-2, 1% grade 3), anorexia (16% grade 1-2, 2% grade 3), and vomiting (14% grade 1-2).

EKB-569: EKB-569 is a selective, irreversible inhibitor of the EGFR, was well tolerated in patients with advanced solid tumors of the colon, lung, breast, head and neck. A phase I study with 30 patients with advanced tumors of different origins established the MTD at 75 mg EKB-569 per day for both cohorts, intermittent-dose schedule (14 d of a 28-d cycle) and continuous-dose schedule (each day of a 28-d cycle) being the DLT grade 3 diarrhea[98]. In a phase Ⅰ/Ⅱa study of EKB-569 in combination with FOLFOX-4 (29 patients), 4 out of 11 patients who completed 4 cycles achieved a PR, 6 patients had stable disease, and 1 patient had progressive disease[99]. Grade 3/4 Toxicity included neutropenia and diarrhea. Moreover, a phase Ⅰ/Ⅱa study of EKB-569 in combination with FOLFIRI (39 evaluable patients out of 47) showed a 38% of RR[100].

CONCLUSIONS

When administered alone new targeted therapies have demonstrated activity in different *in vitro* and *in vivo* studies. However, the clinical use in patients when administered as a single agent is not so brilliant. On the other hand the combination of these drugs with classical chemotherapies has shown better clinical profiles reflected in an improvement in OS and PFS. The FDA approved Cetuximab as a second line therapy in combination and Panitumumab has also been approved as a second and third line therapy for advanced CRC patients. An important number of clinical trials with second or first generation of TKIs is ongoing. Perhaps the role of TKIs in mCRC patients is maintenance treatment in individuals with objective response or stabilisation of their tumor.

There is also the challenging possibility of combining different targeted therapies in order to overpass tumor resistance. Combining targeted therapies against different pathways is also a possibility. The cross-talk at a molecular level of the different networks implicated in cell biology is almost unknown. However there are more data that implicate different molecular networks when studying resistance to targeted therapies against one pathway.

All these data must encourage clinicians and basic researches to hold on in their efforts of untangling the network behind EGFR trying to transform all that effort in improving patients quality of life as well as improving survival There are different clinical scenarios in our patients and each of them should have its own solution. In some cases the approach will be combining chemotherapy with targeted therapy, targeted therapy with radiotherapy or even targeted therapy alone. In anyway we have still a lot of clinical trials to start and new drugs to be tested in order to find the adequate solution for each of our patients.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Pharmacogenomics in colorectal cancer: The first step for individualized-therapy

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Abstract

Interindividual differences in the toxicity and response to anticancer therapies are currently observed in practically all available treatment regimens. A goal of cancer therapy is to predict patient response and toxicity to drugs in order to facilitate the individualization of patient treatment. Identification of subgroups of patients that differ in their prognosis and response to treatment could help to identify the best available drug therapy according the genetic profile. Several mechanisms have been suggested to contribute to chemo-therapeutic drug resistance: amplification or overexpression of membrane transporters, changes in cellular proteins involved in detoxification or in DNA repair, apoptosis and activation of oncogenes or tumor suppressor genes. Colorectal cancer (CRC) is regarded as intrinsically resistant to chemotherapy. Several molecular markers predictive of CRC therapy have been included during the last decade but their results in different studies complicate their application in practical clinical. The simultaneous testing of multiple markers predictive of response could help to identify more accurately the true role of these polymorphisms in CRC therapy. This review analyzes the role of genetic variants in genes involved in the action mechanisms of the drugs used at present in colorectal cancer.

Key words: Colorectal cancer; Pharmacogenomics; Chemotherapy; Polymorphisms; Markers

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INTRODUCTION

Colorectal cancer (CRC) is the second most prevalent cancer and the third leading cause of cancer death worldwide with almost 500 000 related deaths every year[1]. Approximately half of all persons develop local recurrence or distant metastasis during the course of their illness, and the median survival time for these patients can vary from approximately 4 to 22 mo. The basis of treatment for metastasis or recurrent colorectal cancer is chemotherapy, although small number of patients can undergo surgery or others forms of loco regional treatment. While the Dukes and Tumor Node Metastasis (TNM) staging system identifies broad patients groups that vary in their long-term prognosis, considerable heterogeneity exists within each of different chemotherapy agents with regard to response to treatment.

The most studied drug in CRC, the antimetabolite 5-fluorouracil (5-FU), was developed over 40 years ago. In the metastasis disease setting, single-agent 5-FU produced response rates of only 10%-20%[2]. Over the last 5 years, the median survival for patients with metastasis colorectal cancer has nearly doubled from 12-22 mo and the combination of 5-FU with new classes of drugs, such as oxaliplatin and CPT-11 (Irinotecan), has significantly improved response rates up into the 40%-50% range in patients with metastasis colorectal cancer[3]. Figure 1 shown chemical structure of these compounds. Furthermore, the use of novel biological agents, such as the monoclonal antibodies Cetuximab (an epidermal growth factor receptor (EGFR inhibitor) and Bevacizumab (a vascular endothelial growth factor (VEGF) inhibitor), have recently been shown to provide additional clinical benefit for patients with metastatic colorectal cancer[4,5].

The objective of pharmacogenomics is to elucidate the complex genetic network responsible of drug efficacy and adverse drug reactions. The ultimate goal is to provide new strategies for optimizing the individual's response to drug therapy based on patient's genetic information[6]. Current methods of basing dosages on weight and age will be replaced with dosages based on an individual's genetics. This will maximize the therapy's value and decrease the likelihood of overdose.

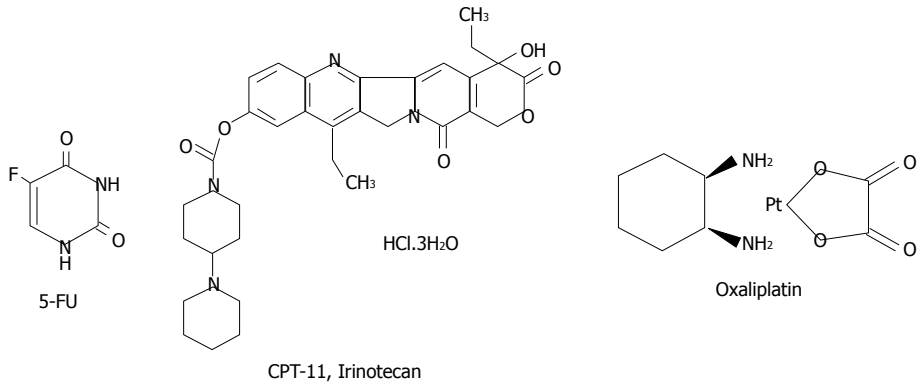

Figure 1 Chemical structure of the three most important drugs used in colorectal chemotherapy: 5-FU, CPT11 and Oxaliplatin.

In CRC, a limited number of predictive markers have been identified to date. The use of these as individual predictive markers has led to somewhat conflicting results. However, if these markers are used in combination they could provide a greater ability to reliably predict response to treatment[7]. Recent advances in our understanding of the molecular biology of CRC should lead to the identification of other panels of potential prognostic and predictive markers.

POLYMORPHISMS AND FLUOROPYRIMIDINES

To this day, the fluoropyrimidines (FPs) including 5-fluorouracil (5-FU), 5'-fluoro-2'-deoxyuridine, capecitabine, tegafur and S1, remain a major component of many standard regimens for numerous cancer types and a baseline component in many experimental regimens with novel agents[8]. Initially, 5-FU was the only effective systemic treatment for CRC, and since leucovorine enhances this effect, 5-FU and LV are given together[9]. FL reduces tumor size by 50% or more in approximately 20% of patients with advanced CRC, and prolongs median survival from approximately 6 mo to approximately 11 mo. When given as adjuvant therapy after the complete resection of tumor that has spread to regional lymph nodes (Stage Ⅲ), FL increases the probability of remaining free of tumor at 5 years from approximately 42% to 58% and the likelihood of surviving for 5 years from 51% to 64%[10].

5-FU, an analog of uracil, is an anticancer prodrug that, after administration, is converted intracellular into three main active metabolites: 5-fluoro-2-deoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). The main toxic effects are mediated by the inhibition of thymidylate synthase (TS) through the formation of an extremely stable ternary complex among FdUMP, TS, and the cofactor 5, 10-methylene-tetrahydrofolate (CH_2FH_4)[11]. The formation of this complex prevents the methylation of the deoxyuridine -5'-monophosphate (dUMP) into deoxythymidine-5'-monophosphate (dTMP) catalyzed by TS. However, the incorporation of the FP metabolites, FdUTP and FUTP, into DNA and RNA respectively, contribute also to 5-FU cytotoxicity[12] (Figure 2).

The common role played by FPs makes stratification according to likely response to this agent a relevant starting point in efforts to individualize treatment. For this purpose, reliable indicators for the prediction of the expected response are required. In the last few decades, intensive research aimed at understanding FP activity and extensive testing of patient's outcomes have highlighted a number of characteristics as potential indicators of response.

Overexpression of TS has been reported in many types of tumors including breast, colon, gastric, and melanoma. In particular, TS overexpression has been found to be significantly associated with a low response to treatment based on 5-FU, both as adjuvant[13] and metastatic therapy[14] Several studies have proposed that genetic polymorphisms of TS gene can affect the response to 5-FU[15-17]. TS expression seems to depend on the number of the so-called TSER, tandem repeat polymorphic copies of 28 bp present in the 5'-promoter enhancer region of the gene[18]. TSER polymorphisms, therefore, are involved in the modulation of TS protein levels and can affect the drug response after administration of fluoropyrimidine. Most Caucasian subjects may be carriers of double (TSER*2) or triple (TSER*3) repetitions for this type of polymorphism, although there have also been reports of sequences with even more copies. An increase in the number of repeats gives rise to an increase in both mRNA and protein TS levels. Three copies of such repeats (TSER*3) lead to a TS expression which is 2.6 times higher than that produced by the presence of only two copies (TSER*2). Patients with CRCs, which show homozygote triple-tandem repeats (3R/3R), present high levels of intratumoral TS mRNA, elevated levels of TS protein, and a lower rate of response to chemotherapy than subjects with CRCs showing homozygote double-repeats (2R/2R)[19]. Similar results have been obtained in patients with metastatic CRCs[20]. Moreover, a study involving 221 Duke's C stage CRC patients has shown that, with regard to survival rate, tumors with 3R/3R genotypes benefit less from chemotherapy than those with 2R/2R and 2R/3R genotypes[16]. A meta-analysis of 20 studies has made it possible to investigate the association between levels of TS expression and the survival of CRC patients[21]. The results have shown that high levels of TS in patients at any stage of the disease are predictive of outcome[22]. However, the predictive role of TS levels in early-stage CRC patients

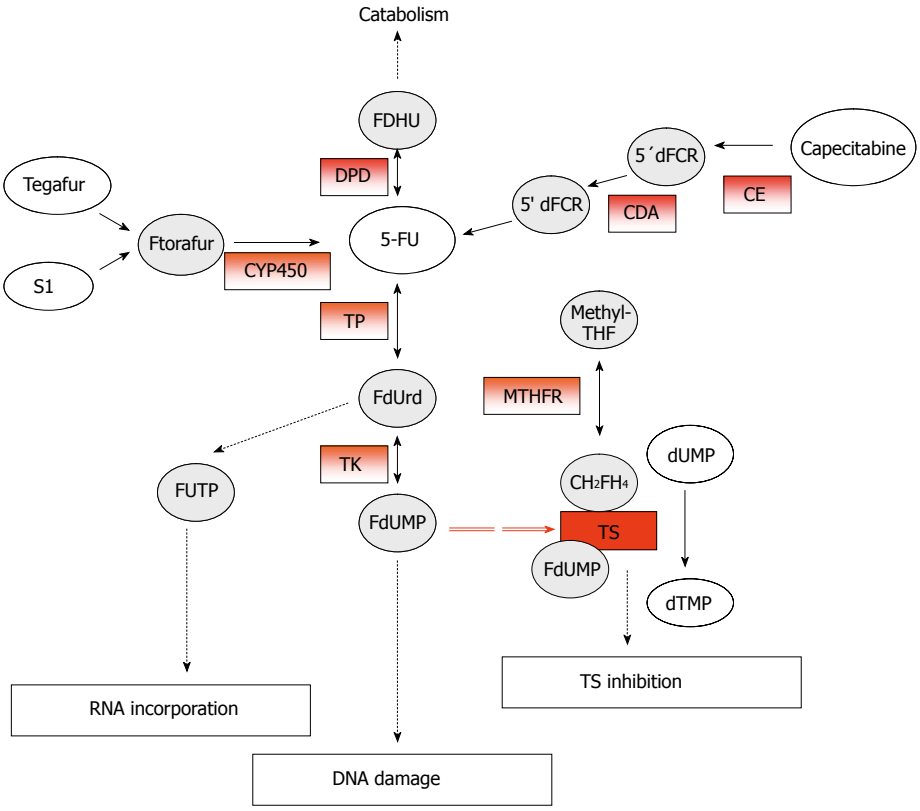

Figure 2 Metabolism and mechanism of action of 5-fluoruracil (5-FU). The potential predictive markers for 5-FU response are in red-boxes.

undergoing chemotherapy is still not fully understood; in fact, whereas in subjects undergoing surgery only, high TS levels are an independent prognostic factor for outcome, in those undergoing surgery and adjuvant FU, TS expression does not seem to predict outcome. Another study reports that in patients with advanced CRC treated with 5-FU/oxaliplatin, intratumoral TS levels appear to have an independent predictive value for survival[23]. Nevertheless, the data so far reported in literature are discordant; although, in fact, TS levels have prognostic value for CRC, this is lower in surgically-treated patients who undergo adjuvant therapy with 5-FU when the TS expression is low, but may be effective for tumors with high TS expression.

TP, also known as platelet-derived endothelial cell growth factor, catalyzes the conversion of 5-FU to the more active nucleoside form and has been shown to be an *in vitro* determinant of 5-FU activity. High expression of either TS or TP in colorectal tumors was shown to be an independent variable so that low expression of both enzymes in tumors predicted a very high expression rate to 5-FU as well as a significantly longer survival, whereas none of the patients with high expression of either TP or TS were responders. These data are in contrast to those demonstrating that cells with higher levels of TP should be more sensitive to 5-FU. These discrepancies may be due to the fact that high TP gene expression was not directly reflected in its protein products, and 5-FU metabolism may be limited by the availability of co substrates, or due to the role of TP as an angiogenic factor.

5-FU is inactivated in the liver by dihydropyrimidine dehydrogenase (DPD), which is the first key enzyme involved in the catabolism of the uracil and thymine into β-alanine. DPD activity is extremely variable in tumoral tissue and this variation might make a difference to the efficiency of 5-FU treatment, since intratumoral drug concentration is one of the most important factors for the determination of the antitumoral effect[24]. Deficiency in DPD activity, however, leads to severe toxicity correlated to 5-FU which may even be fatal. The partial or total lack of this enzyme has, in fact, been associated with severe toxicity (mucositis, granulocytopenia, and neuropathy), and in several cases even death, after 5-FU administration[25]. Analysis of the prevalence of various genetic variants of DPD among patients with DPD deficiency has shown that the most common mutation in DPYD is a G-A transition at the invariant GT splice donor site flanking exon 14 (IVS14 + 1G > A) in Caucasian populations; this mutation is responsible for the lack of exon 14 in mRNA transcript resulting in production of a truncated mRNA with virtually not present enzyme activity[26]. This allele is known as DPYD*2A and is one of the variants associated with severe toxicity after 5-FU treatment[27]. Recently two new missense mutations have been identified on codon 496 (A→G) in exon 6 and on codon 2846 (A→T) in exon 22, the latter in a patient with a total lack of DPD[28].

In the last few years, with the recognition that CH_2FH_4 was essential for the formation of the FdUMP-TS ternary complex, folate metabolism has also begin to emerge as a focus for FP response prediction. MTHFR converts CH_2FH_4 to 5-methyltetrahydrofolate. Consequently, it could be expected that the functionally comprised C677T variant would lead to increase CH_2FH_4 concentrations and thereby enhanced FP activity. Further support of a role for folate metabolism in determining FP response has been provided by the observation of a survival benefit from 5-FU treatment for colorectal cancer patients with DNA

hypermethylation. Higher levels of folate intermediates, including CH_2FH_4, have been demonstrated in tumors with DNA hypermethylation[29]. Cohen and colleagues[30] found a statistically significant trend towards increased response to fluoropyrimidine-based chemotherapy with increasing copy number of the MTHFR 677 T allele in a study of 43 patients with metastatic colorectal cancer. In contrast, Wisotzkey and co-workers[31] did not observe a difference in survival by MTHFR C677T genotype among 51 Stage Ⅲ colon cancer patients treated with 5-FU. However, both studies had a small number of subjects with the MTHFR 677TT genotype (n = 5), and lacked adjustment for potential confounding factors such as primary tumor site or type of chemotherapy received. Only one study has evaluated the effects of the MTHFR C677T, A1298C and TSER genotypes on time to progression and response to 5-FU-based treatment. Jakobsen and co-workers[32] studied 139 patients with metastatic colorectal cancer being treated in a randomized trial comparing three different 5-FU dosage levels. A greater percentage of individuals with the TSER 3R/3R or MTHFR 677T genotypes responded to treatment, and these same individuals had a statistically significant increase in time to disease progression for the first 8 mo post-treatment. However, later in the course there was no statistically significant difference in time to relapse by MTHFR or TS genotype.

Treatment of metastatic CRCs now includes the use of another chemotherapeutic agent, Capecitabine, which is an oral precursor of 5-FU. Due to its poor bioavailability and rapid catabolic clearance by DPD, 5-FU is unsuitable for oral delivery. Capecitabine or Xeloda® is a rationally designed oral fluoropyrimidine carbamate that, after selective conversion to 5-fluorouracil within solid tumors, acts by inhibiting thymidylate synthase activity. This would theoretically yield two advantages, enhanced drug concentrations at the tumor site and thus greater antitumor activity, and reduced drug levels in normal tissues with a consequent reduction in systemic toxicity.

Capecitabine is well absorbed by the gastrointestinal tract and undergoes a three-step enzymatic conversion to 5-FU. First metabolized in the liver by carboxylesterase to 5'-deoxy-5-fluorocytidine, capecitabine is converted in the liver and tumours tissues by citidine deaminase to 5'-deoxy-5-fluorouridine. A tumor-selective phenomenon is facilitated by higher intra-tumoral levels of thymidine-phosphorilase, the enzyme responsible for the final conversion step to 5-FU. With regard to 5-FU, low levels of TS and DPD lead to a better response to capecitabine. In particular, it has been observed that 75% of metastatic colorectal cancer patients, with homozygote double-repeat variants in TS (2R/2R), respond better to capecitabine administration compared with 8% of those with hetero-zygote variants (2R/3R) and 25% of those with triple-repeat homozygote variants (3R/3R)[33].

Recent advances in our understanding of the molecular biology of CRC should lead to the identification of other panels of potential prognostic and predictive markers associate with colorectal carcinogenesis.

In CRC, genetic instability has been recognized as a factor in the origin of malignant lesions, resulting in clonal evolution of genetic events acquired in the course of tumor progression. Microsatellite instability (MSI) is common to many forms of cancer and is found in 10%-14% of sporadic colon cancers[34]. MSI is caused by mutations in the mismatch repair (MMR) genes, such as hMSH2, hMLH1 and hMSH6, resulting in failure of the DNA MMR system to correct errors that occur during replication. An *in vitro* study[35] demonstrated that restoration of hMLH1 activity in the MMR-deficient HCT116 cells increased their sensitivity to 5-FU. Various studies have investigated the prognostic role of MSI in Stage Ⅱ CRC. The studies have confirmed a consistent and independent association between MSI-high (MSI-H) phenotype and superior survival in Stage Ⅱ and Stage Ⅲ CRC patients[36]. Furthermore, Lim *et al*[37] demonstrated that patients with MSI tumors exhibited better recurrence-free survival compared with those with microsatellite stable (MSS) tumors. Moreover, the use of adjuvant chemotherapy did not benefit these patients. The use of MSI as a predictive marker of response to adjuvant chemotherapy still remains controversial. On the other hand, it has been reported that 70% of colorectal cancers have lost a portion of chromosome 17p, or 18q or both. The 17p chromosome contains the p53 gene, which is an important tumor suppressor, and is reported to be mutated in 40%-60% of colorectal cancers[38]. p53 status has been studied as a prognostic factor, and more recently as a predictor of response to cancer chemotherapy[39]. The study published by Tang and colleagues describe that p53 mutation was associated with a poorer prognosis in Stage Ⅱ and Ⅲ CRC patients who received surgery alone, whereas p53 was not a prognostic factor among those patients who had received 5-FU-based adjuvant chemotherapy[40]. However, Ahnen and co-workers found that patients with Stage Ⅲ CRC, whose tumors overexpressed p53, did not derive significant survival benefit from adjuvant 5-FU-based treatment[41].

POLYMORPHISMS AND IRINOTECAN

The combination of 5-FU together with other drugs such as Irinotecan (CPT-11) has led to promising results in the treatment of CRCs, particularly in first line therapy of patients with metastatic disease. Partly as a result of the development of this agent, survival of patients suffering from incurable colorectal cancer has doubled during the last decade[42]. Like other camptothecins, the anti-neoplastic agent irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin) and in particular its active metabolite SN-38 (7-ethyl-10-hydroxycamptothecin) stabilize the DNA-topoisomerase I complex by binding to it, preventing the resealing of single strand breaks[43]. Irinotecan prevents the replication division to proceed which results in double strand breaks and ultimately in its anti-tumor effect and its characteristic adverse effects on rapidly dividing tissues, such as bone marrow and intestinal mucosa. The main dose-limiting toxicities of irinotecan therapy are therefore myelosuppression and delayed-type diarrhea[44,45].

In humans, irinotecan is hydrolyzed into its active metabolite SN-38 by carboxylesterases, present in serum, intestines, tumor tissue, and in high content in

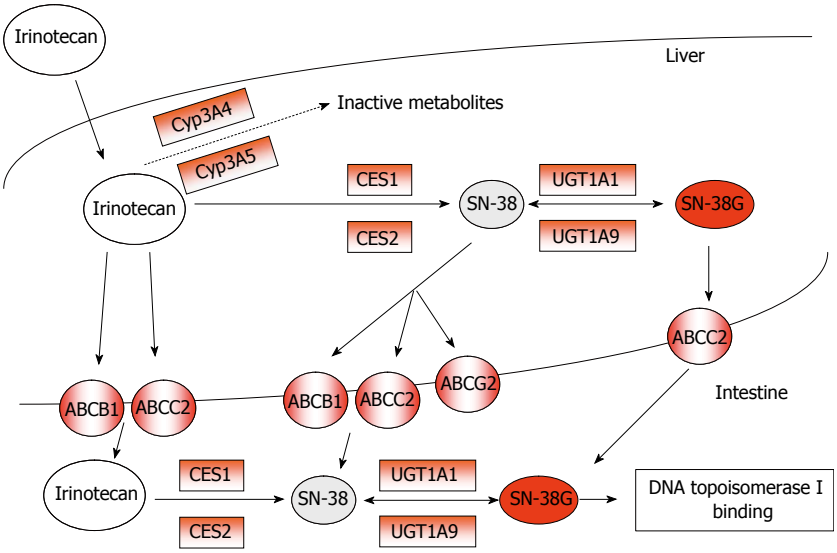

Figure 3 Metabolism and mechanism of action of Irinotecan (CPT-11). The potential predictive markers for CPT-11 response are in red-boxes.

the liver[46]. Recently, the opinion is emerging that intratumoral activation of irinotecan into SN-38 by CES might be even more important than systemic circulating SN-38 levels, formed by hepatic CES[47]. Although plasma levels of SN-38 are relatively low, relations between SN-38 and myelosuppression and/or diarrhea have been demonstrated[48]. Uridine diphosphate-glucuronosyltransferase 1A (UGT1A) mediated glucuronidation of SN-38, forming a β-glucuronic acid conjugate (SN-38G; 10-O-glucuronyl-SN-38), is the main pathway of detoxification for SN-38. Irinotecan is also sensitive to cytochrome P450 3A (CYP3A) that mediated oxidative pathways, resulting in the formation of inactive metabolites. Moreover, irinotecan, SN-38, and their metabolites are excreted by drug-transporting proteins from the adenosine-triphosphate binding cassette (ABC) transporter superfamily[49] (Figure 3).

The CES genes, located on chromosome 16q13-q22, are supposed to be highly conserved during evolution. However, recently, several polymorphisms in the CES-genes have been described, some of which with major racial differences in distribution[50]. Although the interpatient variation in CES activity is high and some SNPs appear to be very common[51], the functional consequences of reported SNPs on the *in vivo* activation of irinotecan into SN-38 are thought to be limited. Marsh *et al*[50] did not demonstrate any functional relationship between the presence of SNPs in the CES genes and CES mRNA levels, except for an intronic SNP (IVS10-88) in CES2 which was associated with reduced CES2 mRNA expression in colorectal tumors, but not in normal colonic mucosa. Neither did Charasson *et al*[52] find any influence of 11 silent SNPs in CES2 on gene expression or functional activity. Lack of association may be explained by the ineffective activation of irinotecan by CES, the role of other esterases, and the complex metabolic pathway of irinotecan. It may also be possible those other proteins regulate CES transcription and translation, or that other factors are rate limiting in the formation of active CES. However, as SNPs in CES may lead to less transcription and thus might lead to diminished local activation of irinotecan and less favorable therapeutic responses, both *in vitro* and *in vivo* functional investigation of SNPs in the CES genes is needed, especially of recently discovered SNPs in CES2.

Members of the cytochrome P450 superfamily are capable to oxidize more than half of all anti-cancer drugs. Especially the CYP3A subfamily, and in particular, the genes CYP3A4, CYP3A5, CYP3A7, and CYP3A43 are the most important. CYP3A4*1B, a SNP in the promoter area of the gene, was thought to be a promising polymorphism for irinotecan pharmacokinetics, partly as a result of its relatively high allele frequency compared to most other CYP3A4 SNPs[53]. However, Garcia-Martin *et al*[54] reported that the presence of CYP3A4*1B did not correlate with low enzyme activity in Caucasians. In a polygenetic approach to assess genotypes from multiple irinotecan pathway genes with irinotecan pharmacokinetics no effect on irinotecan pharmacokinetics was seen, neither for this SNP nor for the other studied CYP3A SNPs (CYP3A4*2, CYP3A4*3, CYP3A5*3 and CYP3A5*6)[55].

The human UGT superfamily has been classified into the UGT1 and UGT2 families, further classified into three subfamilies (UGT1A, UGT2A, and UGT2B)[56]. All nine functional members of the UGT1A subfamily are encoded by a single gene locus, the UGT1A locus on chromosome 2q37. Especially the UGT isoforms 1A1, 1A7 and 1A9 are involved in the phase Ⅱ conjugation of SN-38 to the inactive metabolite SN-38G[57]. UGT1A1 and UGT1A9 are highly expressed in the gastrointestinal tract and the liver; the primary organ involved in the detoxification of irinotecan. Polymorphisms, resulting in absent or very low UGT1A1 activity, have been associated with three heritable unconjugated hyperbilirubinemia syndromes: Crigler-Najjar syndrome type 1 and 2[58], and Gilbert's syndrome[59]. Gilbert's syndrome is common among Caucasians and is associated with the presence of an extra, seventh, dinucleotide (TA) insertion (UGT1A1*28) in the $(TA)_6$TAA-box of the UGT1A1 promoter region, leading to a considerable reduced enzyme expression of about 30%-80%. The UGT1A1 activity appears to be inversely related to the number of TA-repeats, varying from 5 to 8.

Studies have shown that the homozygous UGT1A1*28 genotype was associated with an increased risk of developing leucopenia and severe delayed-type diarrhea after treatment with irinotecan. Ando *et al*[60] analyze the association between UGT1A1 variants and irinotecan toxicity, revealing in a multivariate analysis that presence of UGT1A1*28 allele was a risk factor for severe toxicity. These data have been confirmed by other groups[9,61]. Based on this knowledge and the finding that demonstrated a good concordance between the UGT1A1*28 genotype and less effective SN-38 glucuronidation prospective studies were initiated. A significant relation was observed between the AUC of SN-38 and the number of TA-alleles[62]. In addition, two other promoter variants (UGT1A1-3279G>T and UGT1A1-3156G>A) have been identified. These variants are in strong linkage disequilibrium with the UGT1A1*28 polymorphism in Caucasians, while this link is less apparent in African-Americans and Asians, suggesting a different haplotype structure among various races[63]. Ando *et al*[64] found a strong a relation for presence of the UGT1A1-3263T>G SNP and the severity of irinotecan induced toxicity, although in a multivariate analysis including UGT1A1*28 as well, this effect was mainly attributed to this latter polymorphism[65]. Presented observations clearly illustrate that UGT1A1 mutations can influence a patient's exposure to SN-38, and, hence, the susceptibility to toxicity. Recently, a study in colorectal cancer cell lines shown that DNA methylation represses UGT1A1 expression and that this process may contribute to the level of tumoral inactivation of the anticancer agent SN38 and potentially influence in clinical response[66].

The adenosine-triphosphate (ATP) binding cassette (ABC) transporters are the largest family of transmembrane proteins that use ATP-derived energy to transport various substances over cell membranes[67]. Their localization pattern suggests that they have an important role in the prevention of absorption and the excretion of potentially toxic metabolites and xenobiotics, including irinotecan and its metabolites.

P-glycoprotein, located on chromosome 7q21, and, among others, expressed in kidney, liver, and intestine, is known for more than 50 SNPs and other polymorphisms in the gene encoding this transporter[68]. Three SNPs which show linkage disequilibrium (ABCB1 1236C>T, ABCB1 2677G>A/T, and ABCB1 3435C>T), have been studied extensively [69]. However, a relation with irinotecan or its metabolites has been not demonstrated in Caucasians. Recently, Balram *et al*[70] showed a relation for ABCB1 3435C>T with irinotecan AUC (area under concentration versus time curves) in a small Chinese population which may be the result of lowered pump activity. In a group of 46 Caucasian patients, a significant effect of the ABCB1 1236C>T polymorphism on the AUCs of irinotecan and SN-38 was seen, resulting in an increase in both AUCs[71]. Although an effect of these three related SNPs on irinotecan pharmacokinetics seems likely, the true clinical relevance of their effects still remains to be clarified.

For the canalicular multispecific organic anion transporter (ABCC2), recently a functional SNP in irinotecan pharmacokinetics has been found (ABCC2 3972C>T). This SNP, studied in 64 Caucasian patients, resulted in highly significant effects on the AUC of irinotecan, and SN-38G, all being higher in patients carrying two 3972T alleles.

In vitro studies have indicated that the irinotecan metabolites SN-38 and its glucuronide conjugate SN-38G are very good substrates for the breast cancer resistance protein[72]. ABCG2, located on chromosome 4q22, was first found to be overexpressed in cancer cells with acquired resistance to anticancer drugs[73]. The ABCG2 gene is supposed to be well conserved and most SNPs found up to now seem unlikely to alter transporter stability or function[74]. Few SNPs with presumed clinical consequence have been studied in relation to irinotecan pharmacokinetics; in particular, a single-nucleotide polymorphism in exon 5 has been described. This ABCG2 421C>A transversion results in an amino acid change of glutamine to lysine at codon 141[75]. Functional consequences of this SNP were demonstrated in Caucasian cancer patients treated with the structurally related camptothecins diflomotecan and topotecan[76]. Patients carrying at least one defective ABCG2 421A allele were found to have higher drug levels. However, in a large group of Caucasian patients pharmacokinetic parameters of irinotecan and SN-38 were not significantly different[77].

POLYMORPHISMS AND OXALIPLATIN

Oxaliplatin (OXA), a third-generation platinum analog that distorts DNA adducts, administered alone or in combination with 5-FU/LV has broaden the therapeutic choices for patients with advanced CRC who may experience hepatic and pulmonary metastasis. The cytotoxic activity of oxaliplatin is initiated by formation of a DNA adduct between the adequated oxaliplatin derivative and a DNA base[78]. Initially, only monoadducts are formed but eventually oxaliplatin attaches simultaneously to two different nucleotide bases resulting in DNA cross-links. The adducts are formed with the N-7 positions of guanine and adenine preferentially and in most cases these reactions result in intrastrand cross-links. In the cell approximately one of every 100 000 bases can be cross-linked by a platinum atom, resulting in 10 000 platinum atoms per cell[79].

In general, the cytotoxic efficacy of platinum compounds in cancer cells can be related to inhibition of DNA synthesis or to saturation of the cellular capacity to repair Pt-DNA adducts. Platinum atoms modify the three-dimensional DNA structure, which inhibits the normal DNA synthesis and repair processes[80].

Interestingly, cellular DNA repair mechanisms seem to differ in their response to Pt or Pt-DACH complexes. After DNA-adduct formation by oxaliplatin, cells will activate cellular repair mechanisms. In general, DNA repair is carried out by specific enzymes that consist of several amino- and sulphur groups. Therefore, oxaliplatin can be covalently bound to these repair enzymes as well, impairing their function[81]. If substantial DNA damage persists this may ultimately lead to the activation of apoptotic pathways and cell death[82].

Several mechanisms are described that confer resistance to oxaliplatin, including diminished cellular drug

accumulation, increased intracellular drug detoxification and increased Pt-DNA adduct repair. However, the overall sensitivity of a cell is multifactorial and the relative importance of each process on ultimate drug sensitivity is difficult to predict[83]. There is growing evidence that common gene variants affect the activity of cellular DNA repair and platinum conjugation.

The uptake of platinum by cells is not completely understood but there is evidence that decreased accumulation is the most common mechanism of resistance to cisplatin[82]. Platinum uptake by cells is an energy requiring process, but it is not saturable and possibly involves transport by a yet unidentified efflux pump. Once inside the cell, conjugation to glutathione (catalyzed by the enzyme glutathione-*S*-transferase, GST) effectively inactivates platinum compounds before DNA damage is induced. This conjugation reaction is followed by cellular excretion and is therefore related to cellular drug resistance as well. A number of studies indicate an important role of GST in oxaliplatin resistance. A single nucleotide polymorphism (SNP) in exon 5 at position 313 (A→G) in the GSTP1 (π) gene results causes the amino acid change Ile105→Val. The mutant GSTP1 (π) enzyme is less potent in detoxification of carcinogens and individuals with two mutant alleles have shown a significant survival benefit from combined oxaliplatin/5-FU treatment[84]. Other common polymorphisms in the GSTT1 (θ) and GSTM1 (μ) genes include deletions that result in complete loss of enzyme activity in homozygous individuals. However, no association with altered survival or clinical response in patients with advanced colorectal cancer treated with oxaliplatin/5-FU was observed for the GSTT1 and GSTM1 genotypes[85].

Since the primary anti-tumor mechanism of oxaliplatin is the formation of Pt-DNA adducts, polymorphisms in genes involving the repair of these adducts, such as nucleotide excision repair, base excision repair, mismatch repair (MMR) and other post-replicative repair pathways, may affect oxaliplatin efficacy. Induction of the enzymes involved in these systems results in increased DNA repair activity, more efficient adduct removal and hence decreased sensitivity to platinum drugs.

Mismatch repair (MMR) is a DNA repair pathway that corrects base mispairs and small strand loops that occur during replication. Loss of MMR function results in an increased spontaneous mutation rate. The MMR system consists of six different proteins, originating from the hMLH1, hMLH2, hPMS2, hMSH2, hMSH3 and hMSH6 genes. *In vitro* studies showed that MMR is not involved in oxaliplatin induced DNA-damage repair, whereas it serves as an important mechanism in cisplatin and carboplatin adduct repair[86]. The conformational distortion of the oxaliplatin DNA complex is different from the cisplatin and carboplatin adduct and this, together with the less polar properties of the DACH-ligand, contributes to a recognition failure of MMR proteins to detect oxaliplatin adducts. To date, no polymorphisms in the MMR pathway genes are known that influence the anti-tumor effects of oxaliplatin.

Single-strand breaks resulting from exposure to endogenously produced active oxygen, ionizing radiation or alkylating agents are repaired by the base excision repair system. X-ray repair cross-complementing group 1 enzyme (XRCC1) contains a domain which functions as a protein-protein interface that interacts with poly (ADP-ribose) polymerase (PARP). Shen *et al*[87] identified three polymorphisms in the XRCC1 gene. One of these, located in exon 10 of this gene, causes the amino acid change Arg399→Gln in the PARP binding domain. The polymorphic enzyme is supposed to be less capable of initiating DNA repair due to altered binding characteristics. In individuals with the mutant Arg399→Gln codon increased DNA damage marker levels are found due to inadequate repair or increased damage tolerance. Patients with at least one of the mutant alleles have a more than five old risk of combined oxaliplatin/5-FU chemotherapy failure compared to patients with two wild type alleles[88].

Nucleotide excision repair is a pathway involved in the recognition and repair of damaged or inappropriate nucleotides. A wide variety of DNA-damage is repaired by NER, including UV-induced photo-products, helix-distorting monoadducts, cross-links and endogenous oxidative damage. At least six proteins are essential for damage recognition and removal by this repair pathway. The first step in this process is recognition of a damaged or inappropriate base by XPA (xeroderma pigmentosum complementation group A protein) and RPA (replication protein A). The adhesion of XPA and RPA to a DNA strand attracts other repair factors to the site followed by enzymatic unwinding of the helix lesion area by XPD. The XPD gene, also known as ERCC2 (excision repair cross complementing group 2), encodes an ATP-dependent helicase that is a component of transcription factor TFIIH. A significant relationship with clinical response to platinum-based chemotherapy was found for the Lys751 →Gln polymorphism of ERCC2[89]. This SNP causes an amino acid change in exon 23 and apparently affecting protein function but not resulting in an alteration of any of the seven helicase domains. Metastatic colorectal cancer patients treated with oxaliplatin/5-FU showed different tumor response for the various genotypes; 24% responders in the Lys/Lys group, *versus* 10% in the Lys/Gln and 10% in the Gln/Gln groups, respectively[90]. Nevertheless, further studies are necessary in order to confim these data and to establish the real importance of polymorphisms in the gene XPD with regard to resistance to platinum agents.

TARGETED-THERAPIES FOR COLORECTAL CANCER

Targeted therapy is defined as a treatment with a focused mechanism that specifically acts on a well-defined target or biological pathway. The ideal cancer target can be defined as a macromolecule that is crucial to the malignant phenotype and is not expressed significantly in vital organs and tissues bind to cancer cells with high affinity and create anti-tumor effects.

In colorectal cancer, two targets, the process of angiogenesis, and the epidermal growth factor receptor, are exploited by the newest monoclonal antibodies that are available for use in CRC patients (Figure 4).

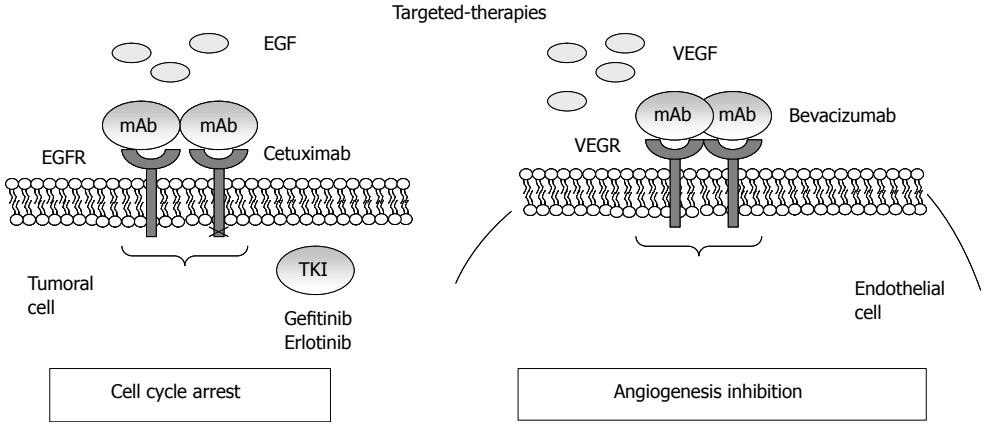

Figure 4 Mechanisms of action for the epidermal growth factor receptor and VEGF.

EGFR-based therapies

EGFR is a tyrosine kinase receptor of the ErbB family that is abnormally activated in epithelial tumors, including 25%-80% of CRCs[91]. EGFR is a 170-kDa cell surface glycoprotein containing three well-identified parts: an extracellular binding domain, a hydrophobic membrane-spanning domain and a cytoplasmic domain containing the tyrosine kinase activity. The bind of specific ligands, EGF and TGFα, to the extracellular domain, leading to dimerization of the receptor with another EGFR (homodimerization) or another member of the EGFR family (heterodimerization). Its activation leads to downstream signaling that stimulates mitogenic and survival pathways such as mitogen-activated protein kinases (MAPKs) and phosphotidylinositol-3 kinase (PI3K)/Akt, which have tumor-promoting activities. Inhibition of these signaling pathways by EGFR antagonists can lead to induction of Bax, activation of caspase-8 and downregulation of Bcl-2 and NF-κB, initiating a cascade of intracellular signaling that ultimately regulates cell proliferation, migration, adhesion, differentiation, and survival[92,93]. Tumor cells that may be activated by ligands such as EGFR and TGFα may then become chemosensitive through EGFR inhibition and activation of these apoptotic pathways.

Agents targeted against the EGFR have been studied extensively in the laboratory, and several have undergone clinical trials, including Cetuximab (Erbitux), a humanized monoclonal antibody directed against the extracellular domain of the EGFR, and the small molecule tyrosine kinase inhibitors (TKIs) Gefitinib (Iressa/ZD1839), and Erlotinib (Tarceva/OSI-774).

Cetuximab binds to the EGFR with high affinity, blocking growth-factor binding, receptor activation, and subsequent signal-transduction events[94]. Preclinical models demonstrated modest *in vitro* and *in vivo* single-agent activity of Cetuximab but significant enhancing activity in combination with cytotoxic chemotherapy[95]. Cetuximab enhanced the antitumor effects of chemotherapy and radiotherapy by inhibiting cell proliferation, angiogenesis, and metastasis and by promoting apoptosis[92]. Several studies have shown that cetuximab is effective in patients with metastasic CRC whose disease has progressed on irinotecan-based chemotherapy. A phase Ⅱ study of cetuximab monotherapy in EGFR-positive advanced CRC patients that failed a previous treatment with irinotecan, obtained 10.5% partial responses and disease stabilization in 35% patients[96]. The result of a multicenter phase Ⅱ study in 246 advanced CRC patients that failed two lines of chemotherapy containing fluoropyrimidines, oxaliplatin and irinotecan have confirmed a partial response of 12% and a disease stabilization rate of 34%. The most important data for the use of cetuximabb, was derived from a large European randomized study, the BOND study, which compared cetuximab with cetuximab in association with irinotecan. Partial response were obtained in 22.9% patients treated with irinotecan plus cetuximab and the time of disease control was 55.5 mo[4].

The development of cetuximab in colorectal cancer was grounded on the premise that EGFR expression by IHC would be prognostic for cetuximab activity, with all trials to date requiring EGFR positivity by IHC. However, Chung *et al*[97] demonstrate no correlation between intensity of EGFR expression and clinical response, challenging this premise. The BOND study results, obtained similar conclusion and the probability or achieving a response was not correlated to the level of EGFR expression in the tumor[4]. On this basis, EGFR-negative colorectal cancer patients would not be excluded from standard protocol treatment with cetuximab on the basis of EGFR status. EGFR analysis by current IHC techniques does not appear to have predictive value, and selection or exclusion of patients for cetuximab therapy on the basis of currently available EGFR IHC does not appear reasonable[98]. This may be due in part to the lack of a standardized protocol and grading system for EGFR expression in clinical samples to technical limitations that are inherent in immunohistochemical methods or, perhaps, to an intrinsically poor correlation between the level of EGFR expression and therapeutic response.

A polymorphic (CA)n dinucleotide repeat is observed in intron 1 of the EGFR gene, which has been shown to be associated with gene expression[99]. It has been demonstrated that as the number of (CA)n repeats increases the level of transcription decreases[100]. However, in CRC cancer, association between the repeat length and EGFR protein expression was not been reported[101]. Neither, polymorphisms of EGFR has been associated with cetuximab therapy.

In addition to cetuximab, several tyrosine kinase

inhibitors have been developed to target EGFR. A recent phase Ⅱ study shown that the combination of capecitabine, oxaliplatin, and erlotinib seems to have promising activity against metastatic colorectal cancer in patients who received prior chemotherapy, with a relatively higher response rate and progression-free survival compared with previous reports of either infusional FU, leucovorin, and oxaliplatin or capecitabine and oxaliplatin in similar patient populations[102].

Skin rash has been the most commonly observed toxicity associated with the various EGFR inhibitors; interindividual differences in the onset, duration and severity of the rash have been observed, and no threshold plasma levels have been linked to the occurrence of the rash. Most intriguing are emerging data demonstrating a significant correlation between skin rash and survival among various patients treated with different anti-EGFR therapies. There are several potential hypotheses being put forward to explain both the variable toxicity and efficacy of EGFR inhibitors. One such hypothesis proposes that variability in clinical observations is related to variable drug exposure. For example, the small-molecule EGFR tyrosine kinase inhibitors gefitinib and erlotinib are metabolized by *CYP3A*, and it is certainly plausible that individuals with variant *CYP3A* alleles might have differences in drug exposure. On the other hand, the previously described CA dinucleotide repeat polymorphism might influence the drug response due to differences in target expression. Data that indirectly lend support to this hypothesis come from a higher response rate observed in Japanese patients compared to Caucasian patients (when treated with gefitinib) two populations with a difference in the frequencies of the *EGFR* dinucleotide repeat variants. However, given the abundant *EGFR* expression in skin tissue, and the observed association between skin toxicity and tumor response; the use of surrogate tissue in this instance might be justified. Nonetheless, this issue highlights an important problem in conducting translational work in this field, since obtaining tumor biopsies in prospective trials for hypotheses generation is not a trivial matter for obvious ethical and practical concerns.

However, robust predictive markers are needed in order to identify the relatively small subsets of patients whose tumours are likely to respond to EGFR-targeted therapies. Candidate markers include phosphorylated EGFR, and phosphorylated effector molecules downstream of the EGFR, such as the mitogen-activated protein kinase (MAPK) and protein kinase B (AKT). However, there are concerns about the stability of phosphorylated proteins in primary tumour samples prior to fixation, and protocols for the collection and processing of clinical material for phosphorylated protein analysis have yet to be validated and standardized. More recently, a work shown that *KRAS* mutation is associated with resistance to cetuximab and a shorter survival in EGFR-positive metastatic colorectal cancer patients treated with this therapy[103]. *KRAS* mutation status might allow the identification of patients who are likely to benefit from cetuximab and avoid a costly and potentially toxic administration of this treatment in nonresponder patients. Prospective randomized study is needed to validate these results that bring a new possibility of targeted therapy adapted to each patient according to its *KRAS* mutation status.

Future issues in the development of EGFR inhibitors include the identification of biologic predictors of response, combination with other targeted agents, and their use in earlier stage malignancies.

VEGF as target for anti-angiogenic therapy

The VEGF family comprises six molecules, the best characterized of which is VEGF-A, which is expressed in at least four isoforms derived by alternative splicing. It is a multifunctional cytokine that acts with receptors expressed on the vascular endothelium to render microvessels hyperpermeable to plasma proteins, alters gene expression, induces endothelial cell migration and proliferation and enhances endothelial cell survival, eventually leading to angiogenesis, permeability and protection against endothelial cell apoptosis and senescence[104,105]. VEGFs mediate their functions by binding to one or more of three tyrosine kinase receptors expressed on endothelial cells: VEGF receptor VEGFR-1 (Flt-1), VEGFR-2 (Flk-1 or KDR) and VEGFR-3 (Flt-4). These receptors have tyrosine kinase activity that initiates intracellular signaling on ligand binding[106]. Other receptors identified (neuropilin-1 and -2) are expressed on numerous cell types, but they do not transmit intracellular signals by themselves after ligand binding[107].

VEGF is a major target for antiangiogenic therapy since its overexpression has been associated with vascularity, endothelial cell migration and invasion, poor prognosis and aggressiveness in most malignancies, including CRC[108]. In CRC, the overexpression of VEGF and its receptor correlated with the development of metastasis[109]. Anti-VEGF strategies include neutralizing antibodies to VEGF or its receptors, ribozymes to receptors and TKIs that block downstream signaling despite ligand binding to VEGFR. Several of these strategies are currently under investigation, including Phase Ⅰ, Ⅱ and Ⅲ trials.

Bevacizumab is a humanized monoclonal antibody that targets and binds to vascular endothelial growth factor-A (VEGF-A), reducing the availability of VEGF and thereby preventing receptor activation[110]. Kabbinavar *et al*[5] reported the first clinical trial of bevacizumab in combination with 5-fluorouracil and leucovorin (5-FU/LV) in previously untreated colorectal cancer patients. Then, different clinical trials shown that Bevacizumab increases survival in association with chemotherapy in the treatment of metastasic CRC. These data led to the FDA approval of bevacizumab for the treatment of metastatic colorectal cancer in February 2004.

As cetuximab, the development of bevacizumab has not included a diagnostic eligibility test and the identification of biomarkers that may predict which patients are most likely to respond to targeted-therapies is of considerable interest. To date, neither direct measurement of VEGF expression nor assessment of tumor microvessel density has been incorporated into the clinical trials or linked to the rates of response to this antibody.

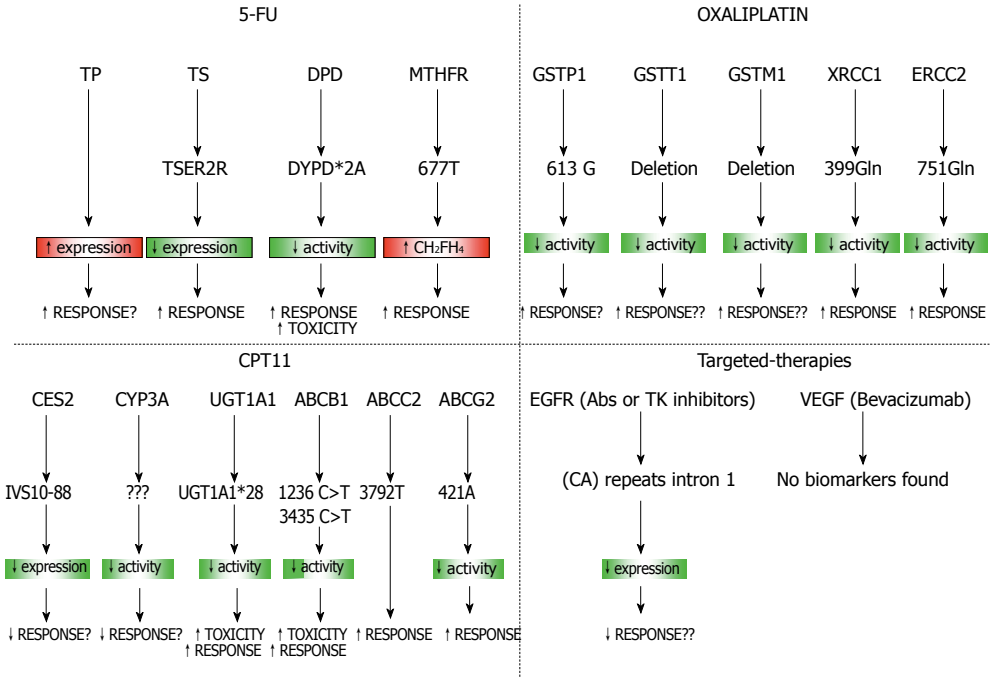

Figure 5 Combination of predictive gene sets for different therapies used in CRC.

Possible biologic surrogates which have been tested in some clinical trials include: DCE-MRI, positron emission tomography scan assessment of tumor blood flow[111], mutations in k-ras, b-raf and p53 genes[112], circulating endothelial progenitors, mature circulating endothelial cells[113], or plasma levels of angiogenic markers, e.g.,VEGF, bFGF. To date, few studies have assessed the potential utility of biomarkers in predicting which patients are more likely to respond to antiangiogenic therapy in the clinic. Tumors may express multiple pro-angiogenic factors and, thus, have different pathways to bypass the VEGF inhibition. Likely, biomarkers that summarize the effects of all angiogenic regulators may better predict patient outcome than the analysis of a single angiogenic factor.

FUTURE PERSPECTIVES

Over recent years, a large number of studies have attempted to define molecular and biochemical markers that may be useful predictors of response to treatment. The introduction of DNA microarray technology has revolutionized our approach to understanding the molecular events regulating the drug-resistant, allowing the simultaneous assessment of thousands of genes. This approach provides a valuable means to identify novel biomarkers of response to treatment as well as novel molecular targets for therapeutic intervention. The candidate gene approach has been widely used to identify the genetic basis for pharmacogenetic traits and becomes increasingly more powerful with the recent advances in genomic technologies. The simultaneous testing of multiple markers predictive of response could help to identify more accurately the true role of these polymorphisms in CRC therapy (Figure 5). High-throughput sequencing and SNP genotyping technologies allow the study of thousands of candidate genes and the identification of those involved in drug efficacy and toxicity. Combination of predictive gene sets identified by gene expression profiling with proteomics and SNPs-array methodologies may enhance the prediction of tumor response to chemotherapy and provide further insights into the molecular characterization of tumor cells. In future studies it will important to combine all these technologies to identify the tumoral response to chemotherapy and finally realize an individualized treatment regimen to each patient.

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TOPIC HIGHLIGHT

Ignacio Gil-Bazo, MD, PhD, *Series Editor*

Novel translational strategies in colorectal cancer research

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Abstract

Defining translational research is still a complex task. In oncology, translational research implies using our basic knowledge learnt from *in vitro* and *in vivo* experiments to directly improve diagnostic tools and therapeutic approaches in cancer patients. Moreover, the better understanding of human cancer and its use to design more reliable tumor models and more accurate experimental systems also has to be considered a good example of translational research. The identification and characterization of new molecular markers and the discovery of novel targeted therapies are two main goals in colorectal cancer translational research. However, the straightforward translation of basic research findings, specifically into colorectal cancer treatment and *vice versa* is still underway. In the present paper, a summarized view of some of the new available approaches on colorectal cancer translational research is provided. Pros and cons are discussed for every approach exposed.

Key words: Translational research; Colorectal cancer; Genomics; Proteomics; Targeted therapies

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INTRODUCTION TO COLORECTAL CANCER

In the current century, despite the recent achievements in the treatment of advanced colorectal carcinoma (CRC), this tumor remains a major public health concern. In fact, it comprises the third most common cancer type to occur in men and women and was the second leading cause of death among cancer patients in the United States during 2006[1].

Different surgical approaches can guarantee low recurrence rates and high survival expectancy in stages I to Ⅲ colon neoplasm patients[2]. Furthermore, adjuvant chemotherapy administration has been shown to effectively improve those rates[3]. However, the subset of stage Ⅱ colon cancer patients to whom adjuvant therapy should be offered is still to be addressed[4]. In fact, different molecular pathology studies and genomic/proteomic investigations are working on that task[5].

In contrast, metastatic colorectal cancer is still far away from being a curable condition and the main goals in the treatment of stage Ⅳ colorectal cancer are to decrease tumor-related symptoms or, alternatively, to prolong symptom-free survival with tolerable toxicity[6,7]. However, the emergence of the highly selective therapeutic antibodies bevacizumab and cetuximab has definitely improved the survival of patients with metastatic CRC[8,9]. This fact has intensively boosted the search for other targeted therapies directed to other fundamental checkpoints in colorectal tumorigenesis[10,11].

Thus, due to colorectal cancer clinical and economic relevance, its basic and clinical research has become one of the most funded among all tumor types in most developed countries. However, the straightforward translation of basic research findings into colorectal cancer therapies is still underway.

In the present paper, a summarized view of some of the new available approaches on colorectal cancer translational research is provided.

TRANSLATIONAL RESEARCH IN CANCER: DEFINING CONCEPTS

Translation of the exciting novel findings made in basic laboratories into testable hypotheses for evaluation in clinical trials is the ultimate aim of translational research in oncology[12-14]. Between a laboratory breakthrough and a real achievement in the clinic, there must be translational research. Thus, the job of the translational researcher is to take the knowledge gained in the laboratory and lay the groundwork needed to develop a new diagnostic tool for a human tumor or a novel drug to be tested in a clinical trial in human beings (Figure 1).

In other words, in order to improve human health,

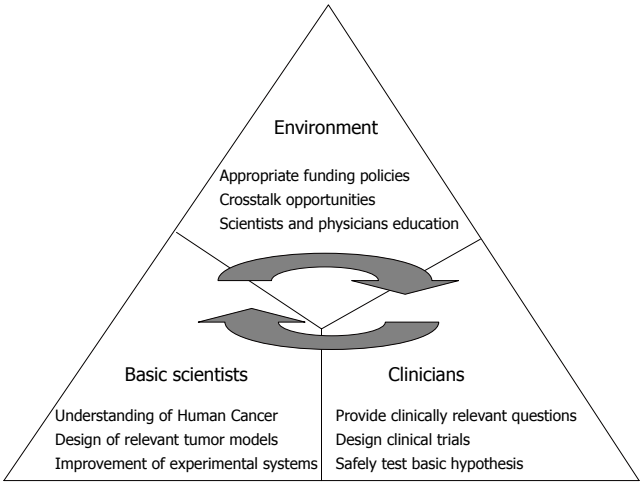

Figure 1 Factors involved in translational research: Interaction between basic scientists, clinicians and the environment.

scientific discoveries must be translated into practical applications. Such discoveries typically start at "the bench" with basic research, in which scientists study disease at a molecular or cellular level[15-19], and then move on to the clinical level, or the patient's "bedside"[20-22]. Scientists are increasingly aware that this bench-to-bedside approach to translational research should really be a two-way highway (Figure 1). Basic scientists provide clinicians with new tools to be used in patients and for assessment of their impact whereas physician-scientists formulate the clinically relevant questions to be tested by basic researchers in a better controlled and more simplified system. Actually, discoveries travel from the clinic to the laboratory in the form of clinical observations, human tissue, diagnostic images, and blood samples, which researchers use to further unlock the molecular and cellular features of cancer (Figure 1).

Often, translational research involves animal studies designed to mimic human conditions[23-26]. Such studies are generally performed with the same care and scrutiny as the best-planned human clinical trials, and comprise a complex set of supporting laboratory techniques that aim to determine how and why the new diagnostic tool or therapy works or fails in these models. Translational research studies may involve many years of investigation on tools and techniques, to try to estimate how safe and how effective the new treatment or diagnostic procedure will be in human trials.

One of the main scopes of translational research in cancer implies the identification and characterization of molecular markers[12]. These can be employed as diagnostic and prognostic tools but also for drug responsiveness assessment or even for targeted therapy design. Molecular markers of tumor responsiveness to drugs would help to select the patient populations that would most likely respond to the drug and identify therapeutic indications. Molecular markers of drug activity in normal tissue would allow pharmacodynamic monitoring of patients that could aid optimization of drug dosing and scheduling to maximize patient response[27]. Furthermore, biological markers involved in tumor initiation and progression can be specifically targeted by new drugs such as therapeutic antibodies[8,9] or anti-tumor vaccines[18].

In fact, another main goal in cancer research is targeted therapy[22]. Translational research is particularly feasible now because of the new understanding of what causes cancer in different individuals, which relates to different combinations of genetic events. This understanding has come primarily from the work of basic research scientists. Until fairly recently, the only effective armamentaria in cancer therapy were surgery, radiation therapy, and chemotherapy. These treatments generally affect neoplastic cells but also non-cancer tissues, leading to the often serious toxicity that characterizes most of traditional cancer treatments[28]. While these standard therapies will continue to play an important role in the treatment of patients with cancer, they can be vastly aided in this process by targeted drugs, which literally target the aberrant molecular pathways that are actually involved in tumor initiation and progression. Therefore, specifically delivering the targeted drug to the malignant cell and its closest environment can significantly relieve cancer treatment related collateral effects[27].

However, since extensive libraries of cytotoxic compounds are being developed for antitumor effect testing, it is becoming more and more common to find new therapies that are successfully developed, tested and commercialized against certain tumors but the ultimate molecular mechanisms involved in tumor response are not clearly known[29]. In those cases, the translational process is rather directed from clinical findings to basic cellular and molecular experiments (from "the bedside" to "the bench"), trying to unravel the complex pathway in which the new compound is playing a definitive role and the specific target or group of them that results inhibited. Therefore, the bidirectional nature of translational research needs to be emphasized[30].

IMPLEMENTING TRANSLATIONAL RESEARCH IN COLORECTAL CANCER

There is still a widening gap between basic research and clinical practice, particularly for colorectal cancer. This might be due to the genetic and molecular complexity of this tumor, the lack of the ideal *in vivo* model for colorectal cancer, and the difficulties found in reproducing animal results into clinical trials in patients.

The principal directions toward which translational research has spread and grown in colorectal cancer in recent years are genomics and proteomics, oncogenic pathways assessment and new targeted therapies discovery (Table 1).

Genomics and proteomics: Searching for new biomarkers and potential target genes

In the last years, there has been an increasingly high effort in the use of genome information in biomedical sciences. This genome information has greatly expanded the insight into the genetic basis of cancer, comprising one of the main fields of interest in translational cancer research. Traditional methods of identifying novel targets involved

Table 1 Translational research technologies in colorectal cancer

Genomics	Proteomics
DNA microarrays	2D-PAGE
	DIGE
	LC-MS/MS
	ICAT
	iTRAQ
	Protein microarrays
	MALDI-TOF
	SELDI-TOF
	Tissue microarrays
Oncogenic pathways	Preclinical models
AS-ODN	Min mice
miRNAs	Msh2, Msh4, Msh6 deficient mice
siRNAs	Apc163 8N mice
	Smad4/Apc mice

in cancer progression were based on studies of individual genes. The following understanding, however, has also shown that gene analysis alone is not sufficient to explain why cancer appears and progresses[31].

Now, the use of DNA microarrays facilitates the analysis of the expression of thousands of genes at the same time and rapidly[32,33]. Microarray analysis has been used for gene expression analysis of different neoplasms[34,35], including CRC[36-39]. However, the application of DNA microarray technology for analysis of CRC is of limited value since it fails to offer direct protein expression measurements[36,40]. In addition, it is already known that important pathways in colon tumorigenesis are regulated at the posttranscriptional level where RNA expression data cannot offer any further information. In fact, due to the alternative splicing of both mRNA and proteins, combined with protein posttranslational modifications, one gene can encode a considerable protein population. Actually, the proteome comprises all proteins that result from the whole genome. In contrast to the genome, the proteome is rather a dynamic parameter constituted by proteins and reflects both the intrinsic genetic program of the cell as well as the impact of its surrounding environment.

However, only a few studies have looked for a further insight into the function and/or importance of individual genes and their application to the proteome research of a tumor. Some of these genes have been proposed as candidate cancer biomarkers[41-43]. More recently a number of proteomic studies have also addressed the identification of potential targets in CRC[44-46].

In the proteomics field, several different technical strategies have been developed and applied to CRC translational research over the last years. Each one has its own advantages and drawbacks that should be considered before deciding the experimental design[47].

The technique leading the field for a long time was the two-dimensional polyacrilamide gel electrophoresis (2D-PAGE)[48]. The 2D-PAGE is based on the separation on a gel of the protein content of a sample in two dimensions according to mass and charge. The gels are stained and spots in samples are compared among different gels. However, a number of serious disadvantages such as its lack of real high-throughput capability (one sample per gel) is responsible for having been replaced by more advanced and capable techniques. Similar to 2D-PAGE, the two-dimensional difference gel electrophoresis (DIGE)[44,46] strengthened the 2D platform by allowing the detection and quantization of differences between three samples resolved on the same gel, or across multiple gels, when linked by an internal standard. Again, it also is a low-throughput technology that does not permit the comparison of many samples in a feasible manner.

Other low-throughput proteomic techniques have recently evolved for cancer protein profiling such as liquid chromatography coupled to tandem mass spectrometry detection (LC-MS/MS)[49], isotope-coded affinity tag (ICAT)[50] and a variation of the latter, isotope tags for relative and absolute quantification (iTRAQ)[51], (both consist of a differential tagging of proteins from samples that are compared using isotope-coded affinity tag in an isotope-dilution mass spectrometry experiment).

A study conducted by Wu *et al*[52] has recently compared some of these diverse proteomic strategies (2D-DIGE, ICAT and iTRAQ) on HCT-116 colon epithelial cells concluding that regarding the number of peptides detected for each protein by each method, the global-tagging iTRAQ technique was more sensitive than the cysteine-specific ICAT method, which in turn was as sensitive as, if not more sensitive than, the 2D-DIGE technique.

Nevertheless, as aforementioned, one of the most important goals in protein profiling in oncology is the discovery of new biomarkers[53]. The use of molecular markers in translational research has expanded considerably during the last 3 decades, and this increased analysis of specific molecular changes has been associated with a concomitant decline in the use of more general and less specific histochemical stains and biochemical assays. Some of the applications for molecular markers include diagnosis, early detection, and prognosis. Also, specific molecular markers are used to study the biology of the disease, to identify targets for novel therapies (e.g., use of Herceptin), and to aid the selection of specific therapies, as previously mentioned.

Therefore, cancer proteomic studies might identify disease-related biomarkers for early cancer diagnosis and new surrogate biomarkers for therapy efficacy and toxicity, but also for guidance of optimal anticancer drug combinations, enabling tailor-made therapy[54]. Furthermore, they could lead to new pharmacological targets. However, a crucial requisite for this purpose is to be able to perform a systematic analysis of a large number of proteins in an easy, reproducible, time-efficient and cost-effective way. High throughput technologies are therefore warranted.

Protein microarrays for instance[55], (targeted proteins bind to spotted probes on a "forward" microarray and specific probes bind to targeted proteins in spotted samples on a "reverse" microarray; bound proteins are detected by direct fluorescent labeling or by labeled secondary antibodies), provide a high throughput approach in terms of number of probes per "forward" array and

number of samples per "reverse" array with the advantage of previously knowing the biomarker identity. On the other hand, the synthesis of many different probes is necessary, the identity of biomarkers has to be known and cross-reactivity of probes along with possible impaired binding of proteins with post-translational modifications (PTM) exists.

In 2002, the Nobel committee acknowledged the advances in mass spectrometry of biopolymers with the recognition of the discovery of electrospray ionization (ESI) mass spectrometry[56,57] and for the discovery of soft laser desorption (SLD) ionization, which led to the development of matrix-assisted laser desorption ionization (MALDI)[58]. These discoveries for peptides, proteins and other macromolecules have been revolutionary, providing easy measurements of molecular weight with unprecedented accuracy. Because the dominant ions generated under SLD and MALDI conditions are singly charged, the technique is most often used in combination with a time-of-flight (TOF) analyzer to extend the m/z range to 100 000 Da and beyond[58]. MALDI-TOF technology is a highly capable tool allowing the measurement of up to 1536 samples per plate, also possessing access to PTM. On the negative side, this technique is unsuitable for high molecular weight proteins (> 100 kDa) and sample fractioning is needed when measuring complex samples.

Surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) technology is a variant of MALDI-TOF in which a selected part of a protein mixture is bound to a specific chromatographic surface and the rest is washed away[47]. Although SELDI-TOF technology only permits 96 samples to be tested by bioprocessor, fractioning of the sample is not necessary and direct application of the whole sample is possible. However, compared to MALDI-TOF, SELDI-TOF provides lower resolution and mass accuracy but requires smaller amounts of starting material. SELDI-TOF is also unsuitable for proteins heavier than 100 kDa.

SELDI-TOF is equally useful for the analysis of cell lysates from cell lines and tissue[59], however, in clinical practice its ultimate value derives from its application to easily accessible body fluids as serum or urine. In fact, in the last years several serum biomarker proteins have been identified through this technical approach[60-62].

In addition, low and high throughput techniques have been shown to be complementary and its combination can lead to a more efficient outcome[63].

In summary, compared to the genome, the proteome provides a more reliable picture of a biological status and is, thus, expected to be more useful than gene analysis for evaluating, for example, disease presence, progression and response to treatment.

A totally different approach for protein profiling has recently emerged in translational cancer research. To evaluate the clinical significance of newly detected potential cancer genes, it is usually required to examine a high number of well-characterized primary tumors. Using traditional methods of molecular pathology, this was a time consuming job that exploited precious tissue resources. However, a high throughput tissue analysis approach, [tissue microarray (TMA) technology], has been developed[64-66]. Using this TMA technology, samples from up to 1000 different tumors are arrayed in one recipient paraffin block, sections of which can be used for all kinds of *in situ* analyses[22,67].

Sections from TMA blocks can then be utilized for the simultaneous analysis of DNA, RNA or protein tumor levels. TMA protein analysis has also been performed in CRC samples for prognostic evaluation[68-72]. However, even though it has been suggested that minute arrayed tissue specimens are representative of their donor tissues, highly heterogeneous cancer types and low levels of protein expression could account for underestimating determined protein expression levels in certain tumors[68,73].

There are multiple different types of TMAs that can be utilized in cancer research including multi tumor arrays (containing different tumor types), tumor progression arrays (tumors of different stages) and prognostic arrays (tumors with clinical endpoints). The combination of multiple different TMAs allows a very quick but comprehensive characterization of biomarkers of interest.

Despite what proteomics have added to translational research in cancer, there are some novel approaches that combine the information provided by genomic and proteomic assays run in parallel in order to complement the translational impact of both procedures[74]. This has also been applied to CRC profiling. Kwong *et al*[75], for instance, studied gene and protein expression performed in parallel across progressive stages of human CRC. For this purpose, they applied cDNA microarray and 2D-PAGE technologies in parallel to analyzed samples collected from 60 CRC cases at various stages of disease progression. Of 47 genes analyzed, 12 (26%) showed significant correlation between mRNA level and protein levels, suggesting that protein abundance is regulated at the transcriptional level. The remaining 31 genes showed either a non-significant correlation between mRNA and protein expression levels or, in 28% of the genes, a negative correlation. Therefore, the authors conclude that posttranscriptional mechanisms play an important role in the regulation of gene products activities in CRC, underline the importance of analyzing gene expression at multiple levels and claim that genomic and proteomic approaches actually complement each other.

In another recent study to identify new biomarkers, Madoz-Gurpide *et al*[76] investigated the feasibility of expressing soluble proteins corresponding to up-regulated genes in surgically resected CRC samples. They used cDNA microarrays (CNIO Oncochip)[77] to identify differentially expressed genes in malignant compared to normal samples isolated from 22 different CRC patients. After investigating different sources of cDNA clones for protein expression, from 29 selected genes, 21 different proteins were finally expressed soluble with, at least, one distinct fusion protein. Additionally, seven of these potential markers were tested for antibody production and/or validation, confirming six of them to be overexpressed in CRC tissues by immunoblotting and TMA analysis[76]. Authors suggest that this kind of approach may provide relevant

biological information of the neoplastic processes and lead to a better characterization of potentially interesting markers in a quite straightforward way for early diagnosis or individualized prognosis assessment.

Oncogenic pathways: Validating target candidates

The previously reviewed development of genomics and proteomics in cancer research has yielded an uncountable number of new potential oncogenic mediators and checkpoints, in CRC, worth further investigating. These novel gene-depending elements, potential new targets for future drugs, are commonly involved in a variety of molecular pathways and their intimate upstream/downstream regulators as well as their crosstalk networks and functional relevance still need to be addressed.

Most widely used experimental methods for molecular pathway research in oncology are performed on fairly well-controlled *in vitro* systems. Recent cell biology achievements and discoveries however, have led to more reliable and physiologically relevant settings where observations on cell behavior and cell fate under particular conditions can be imported into *in vivo* experiments employing animal cancer models and even translating findings into new human therapeutic trials.

In the last few years, several approaches to find molecules able to inhibit the expression of genes (so-called gene-silencing molecules) involved in colorectal cancer progression and therapeutic resistance have been pursued. Sequence-specific gene suppression strategies using antisense oligonucleotides (AS-ODN), ribozymes and deoxyribozymes were initially described and developed[78-82]. AS-ODN derivatives, depending on their type, recruit RNase H to cleave the target mRNA or inhibit translation by steric hindrance. Ribozymes though, directly bind to RNA *via* Watson-Crick base pairing and cleave the phosphodiester backbone of the RNA target by transesterification. Similarly, deoxyribozymes also bind to their RNA substrates *via* Watson-Crick base pairing and specifically cleave the target RNA.

Currently, in addition to their value in target validation studies, different AS-ODN strategies are under evaluation in phase Ⅱ and Ⅲ clinical trials, particularly in hematological malignancies, malignant melanoma and prostate cancer[83,84]. However, consolidating AS-ODN as a broadly applicable functional genomic and therapeutic tool has proven difficult. For instance, difficulties in delivery of the AS-ODN into target tissues, instability of AS-ODN *in vivo*, poor oral availability, uncertainties about the precise mode of action, and toxic effects in animal and human studies have been argued[80,83]. Moreover, a number of class effects are observed with AS-ODN that are unrelated to the specific targeted mRNA sequence. Acute effects include activation of the alternative complement pathway and inhibition of the intrinsic coagulation pathway. In fact, given repeated doses of AS-ODN to animals, accumulation of AS-ODN and/or metabolites occurs in the form of basophilic granules in various tissues, including the kidney, lymph nodes and liver. Although several approaches are known to overcome some of these difficulties[85], very few contributions have firmly supported the use of AS-ODN technology in CRC research[86-88].

But in the field of gene-silencing molecules, the most recent and fascinating tools discovered for studying gene regulation and gene expression control are microRNAs (miRNAs) and small interfering RNA (siRNAs). miRNAs and siRNAs are typically 21 to 25 nucleotide RNA molecules that induce gene silencing by RNA interference (RNAi)[89-91]. Since the description of RNA interference (RNAi) in 1998[92], this gene-silencing technology has been developed into a widely used methodology in basic as well translational research. RNAi was originally discovered as a naturally occurring pathway in plants and invertebrates[92]. Once long double-stranded RNA molecules are inserted into these organisms, they are processed by the endonuclease Dicer into siRNAs. These siRNAs are subsequently incorporated into the multicomponent RNA-induced silencing complex (RISC), which unwinds the duplex and uses the anti-sense strand as a guide to look for homologous mRNAs and degrade them, as previously reviewed by others[93,94]. More strikingly, synthetic short siRNAs (20-25 bp) can be either delivered exogenously or expressed endogenously from RNA polymerase Ⅱ or Ⅲ promoters (in the form of siRNAs or short hairpin (sh)RNAs that are processed by Dicer into functional siRNAs) and used as a new powerful technology for achieving specific down-regulation of target mRNAs in mechanistic research or even therapeutic development in CRC[11,95-98].

Testing targeted therapies: Preclinical modeling in colorectal cancer

Once potential targets are discovered and their expression is successfully inhibited *in vitro*, the safety, efficacy and feasibility of their inhibitors need to be evaluated in animal models in which human disease can be faithfully reproduced. In fact, in the last years, the need of relevant *in vivo* models in colorectal cancer research has prompted many investigators to work on developing reliable, reproducible and human colorectal cancer-mimicking animal models[25,99,100].

However, in colorectal cancer, much has been learned from human inherited syndromes, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC)[101-103]. That knowledge in fact, has been translated into the design and development of CRC animal models.

Although several rat models have been created for the study of colorectal cancer[104-106], in this review, we will focus our attention on mouse models which have profusely evolved in the last few years because of their abundant genetic/genomic information, and easy mutagenesis using transgenic and gene knockout technology. Genetically engineered mice have become essential tools in both mechanistic studies and drug development in CRC, as previously reviewed by others[107]. In fact, mice provide unique opportunities to define and identify genes that are involved in colorectal cancer progression.

The first mouse model obtained to carry a mutation in the adenomatous polyposis coli (APC) tumor suppressor gene was named multiple intestinal neoplasia (Min)[108].

The Min mutation results in a truncated protein and induces the development of multiple intestinal adenomas (even more than one hundred) and a reduced lifespan of on average 150 d in heterozygous mice. Posterior models carrying mutations in different APC alleles have also been developed and each one possesses its own clinical manifestations. However, the majority of them shows small intestine adenomas and colonic tumors and distant metastases are rarely observed. Interestingly, it has been shown that different mutations in the APC gene, in *Apc1638N* mice for instance, confer distinct tumor susceptibility phenotypes and that fact resembles the heterogeneity observed in human FAP families[109]. Other models of hereditary non-polyposis colorectal cancer (HNPCC) have been developed through the mutation of several mismatch repair genes. One representative example are Msh2 deficient mice that are fertile and develop normally, however, these animals develop T-cell lymphomas early in their life and die because of the disease. Msh2 deficient mice that survive more than 6 months develop gastrointestinal adenomas, carcinomas and skin tumors and can also be used for tumorigenesis studies[110].

Finally, other more recent models have also been developed to better study colorectal cancer. Smad4 heterozygous mice bearing *Apc* mutations present an enhanced progression and a more malignant phenotype[111]. Other combinations responsible for increased gastrointestinal tumorigenesis are APC and oncogenic KRAS that seem to be synergistic in enhancing Wnt signaling[112].

CONCLUSIONS

Translational research is a key developing field in biomedicine. The direct application of basic research findings to the patient's diagnosis and treatment is even more important in cancer. In addition, clinical observations can dramatically contribute to basic research improvement and relevant enhancement. Colorectal cancer, due to its epidemiological importance and economic impact, is one of the main entities in which translational research is a reality today.

However, there still is a long way to go until basic researchers and clinical investigators share information and work together in colorectal cancer research on a daily basis.

Several new technologies and tools have demonstrated a great value in cancer and are in fact responsible for the last crucial pieces of research work allowing a new conception of cancer diagnosis and treatment. Among them, the development of new biomarkers for colorectal cancer combining proteomics and genomics is especially relevant.

Also, anti-sense strategies have recently opened the path for new target-specific therapy development. These new therapeutic discoveries need to be tested in preclinical animal models.

Since extensive validation of the above mentioned research fields is necessary, adequate funding is required. This may imply some adjustments in the current funding policy because it involves non-innovative studies. Furthermore, the pool of researchers/clinicians capable of performing translational research must be increased. Additionally, there should be an enhanced participation of patients in clinical trials and an optimization of the efficiency of these trials using validated surrogate markers. Only when these conditions are fulfilled the 'post-genomic' era of biomedical research will have unprecedented opportunities to innovate and improve therapy for cancer.

COMMENTS

Background

In the present paper, a summarized view of some of the new available approaches on colorectal cancer translational research is provided. Translational research in colorectal cancer comprises the identification and characterization of new molecular markers and the discovery of novel targeted therapies. The better understanding of human cancer and the design of more reliable tumor models and more accurate experimental systems is also part of translational research in cancer.

Research frontiers

The principal directions toward which translational research has spread and grown in colorectal cancer in recent years are genomics and proteomics, oncogenic pathways assessment and new targeted therapies discovery.

Innovations and breakthroughs

To our knowledge, there is no other published paper specifically focused on translational research in colorectal cancer. Therefore, we consider this review as a unique and inspiring one.

Applications

The main objective of this manuscript is to help scientists and physicians working on colorectal cancer determine which findings have been already achieved and which others are still underway and provide a better knowledge of new tools and techniques available for this purpose. This focus might inspire other authors in their own research projects and emphasize the need of a new approach to colorectal cancer research.

Terminology

Translational research: Investigation directed to the link of basic and clinical research in order to better define aims and better control tools and experimental systems. Genomics: Part of the bioscience that studies the genome and its implications in disease appearance, progression and response to treatment. Proteomics: Part of the bioscience responsible for peptide and protein investigation and their role in the diagnosis, treatment and research of disease. Targeted therapies: Group of drugs specifically designed to a certain target of the tumor cell such as growth factor receptors, membrane proteins and others.

Peer review

This manuscript is a very good and complete review of the topic exposed.

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Targeting hepatitis B virus antigens to dendritic cells by heat shock protein to improve DNA vaccine potency

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Abstract

AIM: To investigate a novel DNA vaccination based upon expression of the HBV e antigen fused to a heat shock protein (HSP) as a strategy to enhance DNA vaccine potency.

METHODS: A pCMV-HBeAg-HSP DNA vaccine and a control DNA vaccine were generated. Mice were immunized with these different construct. Immune responses were measured 2 wk after a second immunization by a T cell response assay, CTL cytotoxicity assay, and an antibody assay in C57BL/6 and BALB/c mice. CT26-HBeAg tumor cell challenge test *in vivo* was performed in BALB/c mice to monitor anti-tumor immune responses.

RESULTS: In the mice immunized with pCMV-HBe-HSP DNA, superior CTL activity to target HBV-positive target cells was observed in comparison with mice immunized with pCMV-HBeAg (44% ± 5% *vs* 30% ± 6% in E: T > 50:1, $P < 0.05$). ELISPOT assays showed a stronger T-cell response from mice immunized with pCMV-HBe-HSP than that from pCMV-HBeAg immunized animals when stimulated either with MHC class Ⅰ or class Ⅱ epitopes derived from HBeAg (74% ± 9% *vs* 31% ± 6%, $P < 0.01$). ELISA assays revealed an enhanced HBeAg antibody response from mice immunized with pCMV-HBe-HSP than from those immunized with pCMV-HBeAg. The lowest tumor incidence and the slowest tumor growth were observed in mice immunized with pCMV-HBe-HSP when challenged with CT26-HBeAg.

CONCLUSION: The results of this study demonstrate a broad enhancement of antigen-specific $CD4^+$ helper, $CD8^+$ cytotoxic T-cell, and B-cell responses by a novel DNA vaccination strategy. They also proved a stronger antigen-specific immune memory, which may be superior to currently described HBV DNA vaccination strategies for the treatment of chronic HBV infection.

Key words: Hepatitis B virus antigen; Dendritic cell; Heat shock protein; DNA vaccine

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection continues to be a major human health problem, and there are about 350 million chronic HBV carriers worldwide[1]. Chronic HBV infection is associated with serious complications as a result of long-term sequelae such as liver cirrhosis or hepatocellular carcinoma[2]. The host immune response to HBcAg and HBeAg appears critical in both viral clearance and clinical resolution. The ultimate objective for rational vaccine design is the induction of pathogen immunity. In laboratory animals, DNA vaccine has proven to be a simple and effective method to generate protective immunity against a variety of pathogens, including HBV[3,4]. DNA vaccination that can induce both cellular and humoral immune responses has become an attractive immunization strategy against chronic HBV infection. Although it is known that DNA applied either i.m. or intradermally is primarily taken up by muscle cells or keratinocytes, it has become clear in recent years that professional APCs are essential for priming naive T cells following DNA injection. Accumulating evidence indicates that dendritic cells (DCs), the most potent APCs, play a critical role in the induction of immune responses by DNA vaccines[5-7]. Thus, enhancement of antigen presentation by DCs is an attractive strategy to increase the potency of DNA vaccines. However, a major problem of DNA vaccines is its limited potency, because only a very limited fraction of injected DNA molecules are taken up by DCs.

Recently, heat shock protein (HSP) was observed to elicit protective immunity to cancers and infectious

agents. The abilities of HSP include: (a) to chaperone peptides, including antigenic; (b) to interact with antigen presenting cells through a receptor; (c) to stimulate antigen presenting cells to secrete inflammatory cytokines; and (d) to mediate maturation of DCs, making them a one-stop shop for the immune system[8]. These properties also permit to use of HSP for developing a new vaccine. HSP has been reported to activate innate immune responses, to mediate the maturation of DCs, to upregulate proinflammatory cytokines[9-12], and to induce specific CTL responses[13,14].

In this study, we describe a novel DNA vaccination strategy to enhance uptake and presentation of antigens by DCs. Specifically, we developed a DNA vaccination based upon expression of the HBV e antigen fused to HSP, which are versatile immune regulators that chaperone antigenic peptides for MHC class Ⅰ and Ⅱ presentation by DCs. After vaccination, DNA is taken up by various cells that produce and secrete the antigen-HSP fusion proteins. The secreted fusion proteins, in addition to inducing B-cells, are efficiently captured and processed by DCs *via* receptor-mediated endocytosis, and then presented *via* MHC class Ⅰ and class Ⅱ molecules. This study demonstrates a broad enhancement of antigen-specific $CD4^+$ helper, $CD8^+$ cytotoxic T-cell, and B-cell responses by this DNA vaccination strategy, which may be superior to currently described HBV DNA vaccination approaches for the treatment of chronic HBV infection.

MATERIALS AND METHODS

Mice and cell lines

The mice used were female C57BL/6 and Balb/c mice, aged 4-5 wk. All mice were maintained in the animal facility at Baylor College of Medicine with approval of the Institutional Animal Care and Use Committee. The tumor cell lines EL-4, Trampc-2, and CT26 were purchased from the ATCC. EL-4 and Tampc-2 cells were cultured in DMEM medium and CT26 cells in RPMI 1640 medium both containing 10% heat-inactivated FBS (GIBCO) at 37℃ in an humidified 5% CO_2 atmosphere.

DNA constructs

The pCMV-HBeAg-HSP construct was generated by inserting HBeA (be derived from the precore open reading frame by cleavage of its C-terminus, nucleotide: 1901-2452 of HBV genome) & HSP-70 (StressGen Biotechnologies, Victoria, British Columbia, Canada) plasmid into a pCMV vector (Invitrogen, Carlsbad, CA,USA) with the cloning site HindIII & XbaI. Two control vectors, pCMV-HBeAg & pCMV-HSP, were also generated.

DNA preparation and immunization

Plasmid DNA was amplified in *Escherichia coli* DH5α and purified using an endotoxin-free purification kit (Qiagen) according to a standard protocol. Concentration was determined using the UV/Visible Spectrophotometer (Pharmacia Biotech) at 260 and 280 nm, and the material was adjusted to a final concentration of 1 mg/mL with endotoxin-free PBS (Sigma) and stored at -20℃. Mice were divided into 4 groups, which were immunized with different DNA vaccines including pCMV-HBeAg-HSP, pCMV-HBeAg, and pCMV-HSP. Controls were injected with PBS (C57BL/6 mice) or pCMV (Balb/c mice). Immunization method: mice were injected s.c. (C57BL/6) and i.m. (Balb/c) in quadriceps with 100 μg of DNA in 100 μL, two inoculations were carried out with an interval of 2 wk. Two weeks after the second immunization blood and spleens were collected, and BALB/c mice were challenged with CT26-HBeAg tumor cells.

Elispot for T-cell response assay

Elispot assays were used as a measure for T-cell response. Ninety-six well filtration plates (Milipore, Bedford, MA, USA) were coated with AN18 (anti-mouse IFN-γ, Mebtech) at the concentration of 10 μg/mL and kept at 4℃ overnight. Splenocytes were cultured in 96-well plates (1×10^6 cells/mL and 2×10^6 cells/mL) with RPMI 1640/10% FBS containing HEPES, 2MC, and NEAAS. Splenocytes from mice of different groups were stimulated with HBeAg class Ⅰ peptide (HBcAg93-100 peptide), HBeAg class Ⅱ peptide (HBcAg 120-131), or HBV protein (HBsAg, 227 amino acids, 24kD) (BD Pharmingen, SD, CA, USA) for comparing the effect of different DNA vaccinations on the T-cell response. The splenocytes derived from mice vaccinated with HBeAg-HSP were also stimulated with Trampc-2 class Ⅰ peptide (P117-139, WT1), CT26 class Ⅰ peptide (peptide AH1), Tyrosinase protein, Tyrosinase class Ⅰ peptide (Ty-4), Tyrosinase class Ⅱ peptide (Ty-5), and PMSA4 class Ⅱ peptide as controls. Proteins were added at a final concentration of 60 μg/mL, peptides at a final concentration of 30 μg/mL. All assays were performed in triplicates. After stimulation for 20 h at 37℃, the plate was washed with PBS and the second antibody (anti-mouse IFN-γ, Mebtech Mab R4-6A2 biotin) was added for a further incubation at 37℃ for 2 h. Avidin-HRP was added for 1 h at room temperature after washing, then 100 μL AEC was added to each well for coloring for 4 min after washing. The reaction was stopped by drying the membrane. The results were sent to Zellnet Consulting, Inc. (NY, USA) for test.

CTL cytotoxicity assays

CTL cytotoxic activity was determined using a ^{51}Cr-release assay. In brief, splenocytes obtained from mice 2 wk after the second immunization were cultured in 24 well plates with RPMI 1640/10% FBS containing HEPES, 2MC, NEAAS, and IL-2 (50 U/mL). Splenocytes were stimulated with HBeAg class Ⅰ peptide (HbcAg93-100 peptide) for 7 d, with changing half of the medium every 2 d. Target cell lines were cultured with IFN-γ (100 U/mL) for 24 h. EL-4 cells were pulsed with HBeAg class Ⅰ peptide and HBV protein (HBsAg, 227 amino acids, 24 kDa) as target cells. EL-4 cells pulsed with HBV non-related class Ⅰ peptide such as CT26 and Trampc-2 class Ⅰ peptides, non-pulsed EL-4 cells, and CT26 cells pulsed with HBeAg class Ⅰ peptide served as controls. All target and control cells were labeled with ^{51}Cr for 90 min. Cells were added to the wells at effector-to-target ratios ranging from 100:1 to 6.25:1 in triplicates. Plates were incubated at 37℃ for 5 h, before supernatants were collected and activity was assessed in a Gamma counter (Beckman, Fullerton, CA, USA).

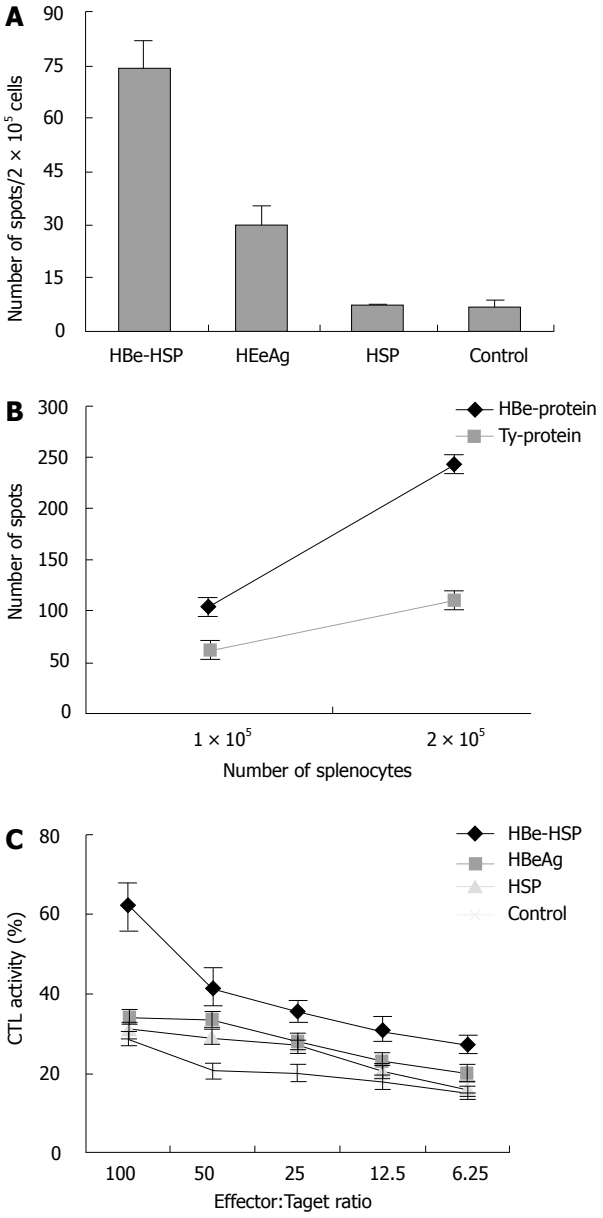

Figure 1 **A**: Comparison of T cell proliferation between different groups after stimulation with HBV protein (C57BL/6 mice); **B**: Comparison of T cell proliferation between stimulation with HBV protein and with non-related protein (Balb/c mice); **C**: Comparison of CTL activity to HBV protein pulsed target cells between different groups (C57BL/6 mice).

Anti-HBeAg antibodies assays

An ELISA assay (BD Biosciences) was used to quantify the antibody response after immunization. Sera were obtained 2 wk after the second immunization. Microtiter plates (Maxisorp) were coated with HBeAg overnight at 4℃. The coated plate was washed with PBS to stop reaction for 1 h at 37℃. Sera were added at different dilutions and the plate was incubated at 37℃ for 2 h. The second antibody was added at 37℃ for 2 h after washing with PBS. Finally, substrate was added and the plate was stored at room temperature for 30 min before stopping reactions with 4N sulfuric acid. Reading of the plate was done in an ELISA reader at 450 nm.

CT26-HBeAg tumor cell challenge test

CT26 cells were transfected with HBeAg using GenePoter reagent (Gene Therapy Systems) according to the manufacture's instructions. Forty-eight hours after transfection, cells were harvested and plated into selective medium in 10 cm dishes, 5×10^4 CT26 cells were plated into 250 μg/mL Geneticin. Two weeks after the second vaccination, 5×10^5 CT26 cells with HBeAg were injected s.c. into mice. Tumor incidence and tumor growth were monitored and tumor size was measured ($v = 1/2ab^2$: v: volume; a: largest diameter; b: smallest diameter).

Statistical analysis

All statistical analyses were performed using student *t*-test. Values of $P < 0.05$ was considered significant.

RESULTS

Enhancement of T cell response and CTL activity by HBeAg-HSP DNA vaccine

To evaluate whether HBeAg-HSP DNA vaccine can enhance immune response *in vivo*, splenocytes were obtained for T cell response and CTL activity. These immune responses were first stimulated with HBV protein and were compared between C57BL/6 and Balb/c mice immunized with different DNA vaccines. In CTL assay, EL-4 cells were pulsed with HBV protein as target cells. ELISPOT showed a stronger T-cell response from the mice immunized with HBeAg-HSP than that from HBeAg immunized mice after stimulation with HBV protein (Figure 1A, spots 74 ± 5 *vs* 31 ± 6, $P < 0.01$). A specific T-cell response was obtained in HBV protein stimulation in comparison with Tyrosinase protein stimulation (Figure 1B). Superior CTL assay to HBV protein pulsed target cell was also observed in mice immunized with HBeAg-HSP in comparison to those immunized with HBeAg (Figure 1C, 46% ± 10% *vs* 35% ± 8% in E: T > 50:1, $P < 0.05$).

We also evaluated the specific stimulating effect of HBV MHC class I peptide on T cell response and CTL activity. After splenocytes were stimulated with HBV MHC class I peptide, ELISPOT showed a stronger T-cell response from mice immunized with HBeAg-HSP than from those which had been immunized with HBeAg (Figure 2A, 76 ± 6 *vs* 29 ± 5, $P < 0.01$). CTL activity to HBV class I peptide pulsed target cells is also stronger in mice immunized with HBeAg-HSP than in mice immunized with HBeAg (Figure 2B, 44 ± 5 *vs* 30 ± 6 in E: T > 50:1, $P < 0.05$). A specific effect on T cell response is also obtained after stimulation with HBV class I peptide in comparison with CT26 class I peptide and TrampC-2 class I peptide stimulation (Figure 2C). A stronger CTL activity to HBV class I peptide pulsed target cells was shown in comparison with target cells pulsed with CT 26 class I peptide and TrampC-2 class I peptide (Figure 2D). T cell response to HBV class I peptide and CTL activity to HBV class I peptide pulsed target cells proved cytotoxic T cell activity.

To evaluate helper T cell activity, the effects of HBV MHC class Ⅱ peptides on T cell response and CTL activity were studied. Splenocytes were stimulated with HBV class Ⅱ peptides for T cell response. A stronger T cell response was obtained from HBeAg-

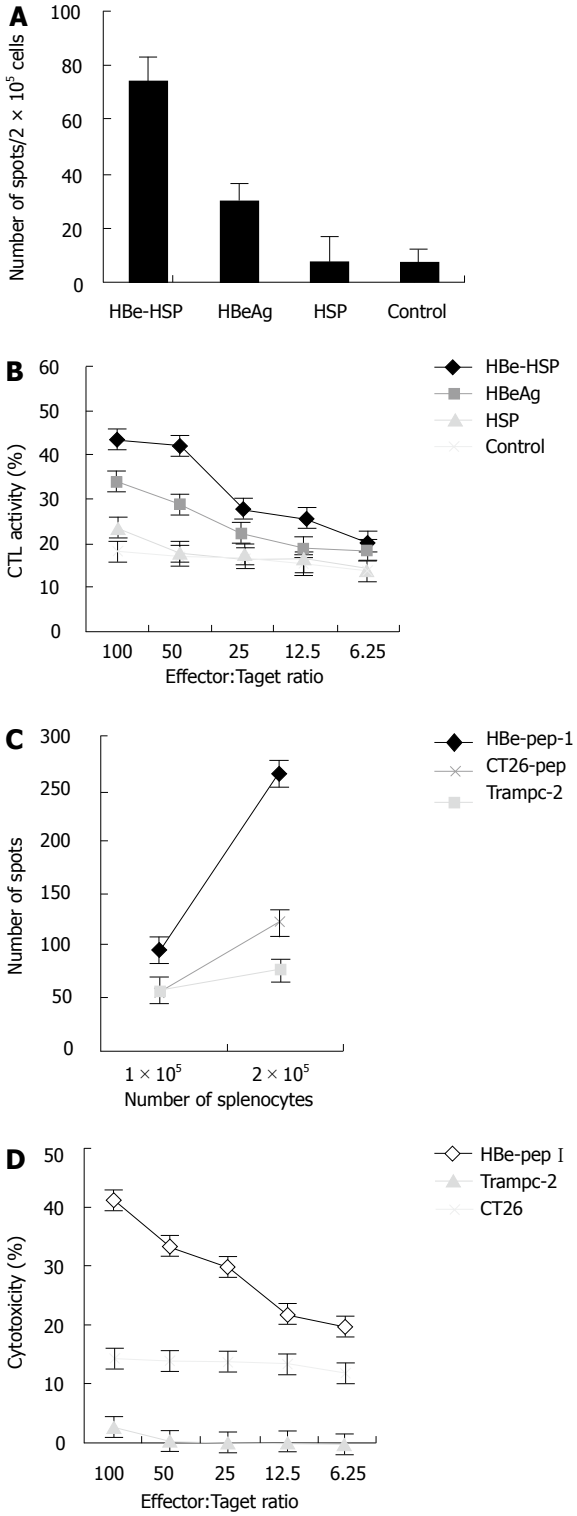

Figure 2 **A**: Comparison of T cell proliferation between different groups after stimulation with HBV class I peptide (C57BL/6 mice); **B**: Comparison of CTL activity to HBV class I peptide pulsed target cells between different groups (C57BL/6 mice); **C**: Comparison of T cell proliferation between stimulation with HBV and with non-related class I peptide (Balb/c mice); **D**: Comparison of CTL activity to HBV class I peptide pulsed target cell between HBV and non-related class I peptide (Balb/c mice).

HSP DNA vaccine immunized mice in comparison with that in mice immunized with HBeAg (A, spots 74 ± 9 *vs* 31 ± 6, $P < 0.01$) and HSP DNA vaccine (Figure 3). A specific stronger T cell response by HBV class Ⅱ peptide stimulation was shown in comparison with that by PSMA4 class Ⅱ peptide, Tyrosin-4 and Tyrosin-5 class Ⅱ peptide stimulation (Figure 3B).

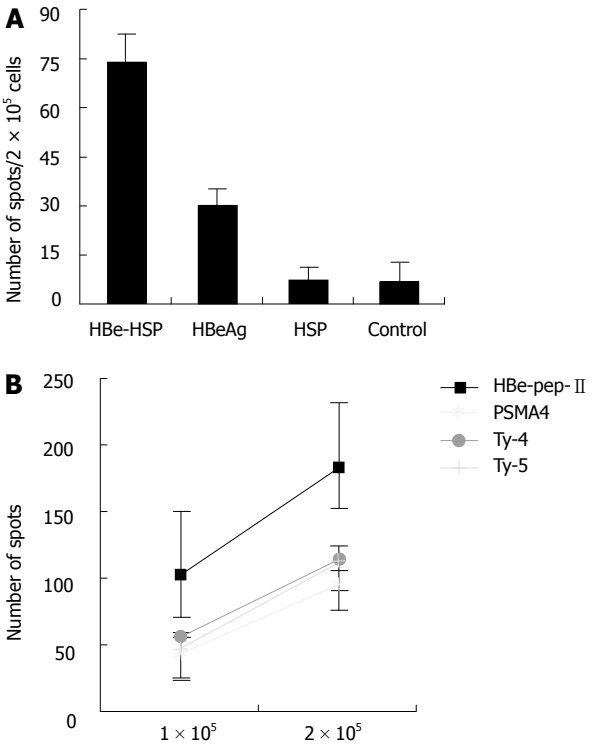

Figure 3 **A**: Comparison of T cell proliferation between different groups after stimulation with HBV class Ⅱ peptide (C57BL/6 mice); **B**: Comparison of T cell proliferation after stimulation with different class Ⅱ peptide (Balb/c mice).

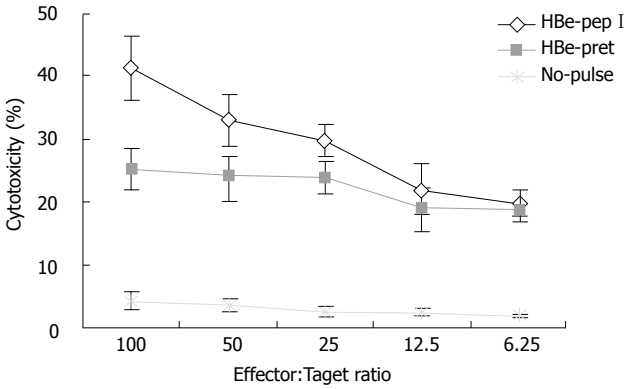

Figure 4 Comparison of CTL activity to target cells pulsed with different agents (Balb/c mice).

We also studied whether HBV related proteins and class I peptides can increase target cell antigenicity. Results suggested that splenocytes from mice immunized with HBeAg-HSP DNA vaccine have a stronger CTL activity to target cells pulsed with HBV protein and HBV class I peptide in comparison to non-target cells and CT 26 cells (Figure 4).

Serum antibody response to HBeAg antigen after DNA vaccination

To determine whether HBeAg-HSP DNA vaccination can also induce an antibody response to HBeAg antigen, we measured serum anti-HBeAg antibody responses by ELISA assay. As shown in Figure 5, antibody levels detected in mice immunized with the HBeAg-HSP DNA vaccine were markedly higher than those immunized with

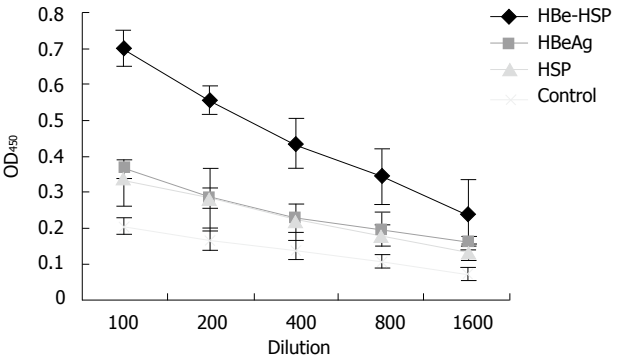

Figure 5 Comparison of antibody titers to HBV between different groups after immunization (Balb/c mice).

the HBeAg or the HSP DNA vaccines ($P < 0.05$). The results indicate the superiority of HBeAg-HSP in inducing humoral immunity.

Systemic immunity enhancement in vivo by HBeAg-HSP DNA vaccine

To evaluate systemic immunity enhancement *in vivo* by the HBeAg-HSP DNA vaccine, Balb/c mice (10 mice/group) were challenged by CT26 cells transfected with HBeAg to observe the antitumor effect after different DNA vaccine immunizations. CT26-HBeAg cells were injected s.c. at 5×10^5/mouse and tumor incidence and tumor growth were monitored. Results showed that there is a low tumor incidence in mice immunized with HBeAg DNA vaccine and a lower tumor incidence in HBeAg-HSP DNA vaccinated mice (Figure 6A), the incidence of tumor are 6/10 in HBeAg-HSP group, 8/10 in HBeAg group and 10/10 in the other two groups. Tumor growth was slowest in mice immunized with the HBeAg-HSP DNA vaccine (Figure 6B). The results suggested that HBeAg-HSP DNA vaccination can induce a stronger immune response to the related antigen.

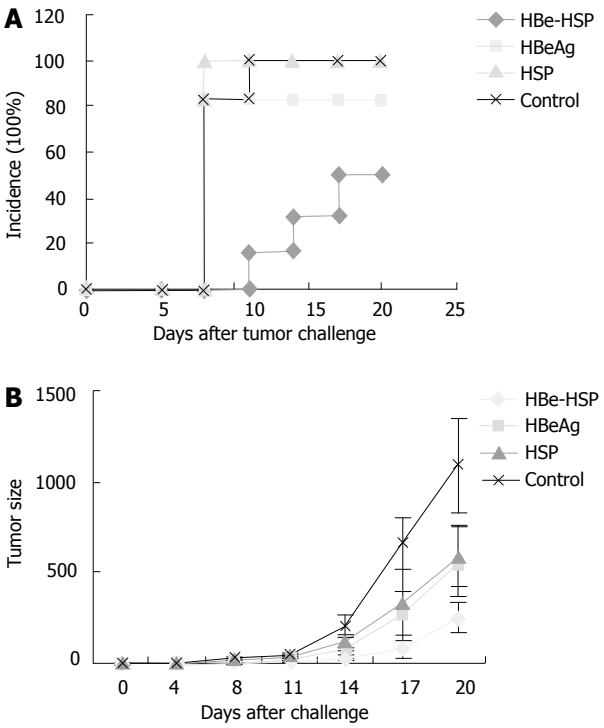

Figure 6 **A**: Tumor incidence after CT26-HBeAg challenge of Balb/c mice; **B**: Tumor growth after CT26-HBeAg challenge of Balb/c mice.

DISCUSSION

HBV infection is a major human health problem and it is associated with a risk of developing liver cirrhosis or hepatocellular carcinoma[1,2]. Thus, effective preventive and therapeutic strategy to chronic HBV infection has been a major exploration[15,16]. Only a small proportion of patients with chronic HBV infection benefit from a treatment with interferon-α (IFN-α)[2]. Antigen-based vaccines have some disadvantages, such as the possibility of reversion to a virulent form, especially in immunocompromised individuals; whole-killed or subunit vaccines do not induce intracellular synthesis of antigen because there is poor or absent presentation of antigen on class I MHC and thus poor induction of a CTL response[17]. DNA-based vaccination is an efficient new technique to stimulate specific immune responses and specific for HBV antigen to induce a strong humoral and cell-mediated immunity against HBV infection[18,19]. HBV(HBsAg, HBc/eAg) DNA vaccine has been popularly studied for prophylaxis or therapy against HBV infection[15,16,20-24].

DNA vaccine has made an attractive alternative to conventional methods of vaccination. HBV DNA vaccination can induce $CD8^+$ T cells as well as a dominant Th1 phenotype among the splenic lymphocytes, so eliciting strong CTL and protective levels of antibody[25-28]. Antigen-presenting cells (APCs) play a key role in induction of immune responses by DNA vaccines. DNA vaccines express native protein antigens in situ which can be recognized by B cells and presented by MHC class Ⅰ and Ⅱ molecules to prime helper T cells and CTLs. Dendritic cells (DCs) are usually thought of as a specific APCs for T cell and B cell activation and regulation of antibody synthesis, presentation of antigen by DCs is a potent stimulus to immune response, particularly to cell-mediated immunity and the development of CTLs. Thus, DCs are critical for initiating and modulating B and T cell responses elicited by DNA vaccination[29-31]. However, only a very limited fraction of injected DNA molecules is taken up by DCs, the intracellular antigens expressed by DCs are difficult to be processed and presented to MHC class Ⅱ[32].

In this study, we designed a novel DNA vaccination strategy to enhance uptake and presentation of antigen by DCs, specifically, we developed a DNA vaccine based upon the expression of the HBV e antigen fused to HSP, which are versatile immune regulators that chaperone peptides for MHC class I and Ⅱ presentation by DCs. The abilities of HSP include: to chaperone peptides, including antigen peptides; to interact with antigen presenting cells through a receptor; to stimulate antigen presenting cells, such as DCs to secrete inflammatory cytokines; and to mediate DC maturation[14]. The HSP70 peptide complex has been shown to elicit $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells and has been used for inducing antitumor immunity and for therapy of infectious diseases[33-37]. The novel vaccination strategy-HBeAg-HSP we developed

has been shown to induce a stronger CTL activity, T cell proliferative response, and antibody response than that of HBeAg DNA vaccine. Moreover, it also showed a stronger anti-tumor immunity to tumor with HBV antigen challenge than that of the HBeAg DNA vaccine. To date, many kinds of cancer vaccines have been tested worldwide and have shown their own advantages. HSP-based cancer vaccine is one of the outstanding representatives[38]. HSP complexes isolated from tumor have been shown to induce specific anti-tumor immunity, HSP alone can also induce non-specific immunity[39]. Recent works by Enomoto and Chan indicated HSP70 based vaccine possess superior properties such as stimulation of DC maturation and T cell proliferation[40,41]. HSP vaccine has been extensively tested in animals and more recently in clinical trials[42,43]. HSP vaccine can induce immune responses against mutated tumor-specific antigens, as well as normal self-antigens. Immune responses to self-antigens by HSP may thus produce damage to normal tissues, however, there are no reports about toxic side effects in mouse models or clinical trials with HSP[44,45].

It is important that exploit of effective DNA vaccination to induce HBV specific immune response to clear HBV infection. The results of this study demonstrate the broad enhancement of antigen-specific $CD4^+$ helper, $CD8^+$ cytotoxic T-cell, B-cell response, and specific anti-tumor immunity by this DNA vaccination strategy, which may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

COMMENTS

Background

Chronic HBV infection is associated with serious complications as a result of long-term sequelae such as liver cirrhosis or hepatocellular carcinoma. The host immune response to HBcAg and HBeAg appears critical in both viral clearance and clinical resolution. DNA vaccination that can induce both cellular and humoral immune responses has become an attractive immunization strategy against chronic HBV infection. However, a major problem of DNA vaccine is its limited potency, because only a very limited fraction of injected DNA molecules are taken up by DCs. In this study, we describe a novel DNA vaccination strategy to enhance uptake and presentation of antigens by DCs.

Research frontiers

A DNA vaccination based upon expression of the HBV e antigen fused to a heat shock protein (HSP) was developed, this study demonstrate that this DNA vaccination strategy may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

Innovations and breakthroughs

DNA vaccine has made an attractive alternative to conventional methods of vaccination. In this study, we designed a novel DNA vaccination strategy to enhance uptake and presentation of antigen by DCs, it may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

Applications

HSP vaccine has been extensively tested in animals and more recently in clinical trials. The results in this study suggested that HBeAg-HSP DNA vaccine may be superior to currently described HBV DNA vaccination for the treatment of chronic HBV infection.

Terminology

DNA vaccine has made an attractive alternative to conventional methods of vaccination. HBV DNA vaccination can induce $CD8^+$ T cells as well as a dominant Th1 phenotype among the splenic lymphocytes, so elicit strong CTL and protective levels of antibody. The novel vaccination strategy-HBeAg-HSP we developed has been shown to induce a stronger CTL activity, T cell proliferative response, and antibody response than the HBeAg DNA vaccine, and it also showed a stronger anti-tumor immunity to tumor with HBV antigen challenge than that of HBeAg DNA vaccine.

Peer review

The novel vaccination strategy-HBeAg-HSP was studied and it is one of the outstanding representatives for the treatment of chronic HBV infection. Moreover, it will be interesting in the treatment of cancer in future. It is deserved to be published.

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CLINICAL RESEARCH

Stability of cirrhotic systemic hemodynamics ensures sufficient splanchnic blood flow after living-donor liver transplantation in adult recipients with liver cirrhosis

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Abstract

AIM: To investigate the correlation between systemic hemodynamics and splanchnic circulation in recipients with cirrhosis undergoing living-donor liver transplantation (LDLT), and to clarify how systemic hemodynamics impact on local graft circulation after LDLT.

METHODS: Systemic hemodynamics, indocyanine green (ICG) elimination rate (K_{ICG}) and splanchnic circulation were simultaneously and non-invasively investigated by pulse dye densitometry (PDD) and ultrasound. Accurate estimators of optimal systemic hyperdynamics after LDLT [i.e., balance of cardiac output (CO) to blood volume (BV) and mean transit time (MTT), defined as the time required for half the administered ICG to pass through an attached PDD sensor in the first circulation] were also measured. Thirty recipients with cirrhosis were divided into two groups based on clinical outcomes corresponding to postoperative graft function.

RESULTS: Cirrhotic systemic hyperdynamics characterized by high CO, expanded BV and low total peripheral resistance (TPR) were observed before LDLT. TPR reflecting cirrhotic vascular alterations was slowly restored after LDLT in both groups. Although no significant temporal differences in TPR were detected between the two groups, CO/BV and MTT differed significantly. Recipients with good outcomes showed persistent cirrhotic systemic hyperdynamics after LDLT, whereas recipients with poor outcomes presented with unstable cirrhotic systemic hyperdynamics and severely decreased K_{ICG}. Systemic hyperdynamic disorders after LDLT impacted on portal venous flow but not hepatic arterial flow.

CONCLUSION: We conclude that subtle systemic hyperdynamics disorders impact on splanchnic circulation, and that an imbalance between CO and BV decreases portal venous flow, which results in critical outcomes.

Key words: Cirrhosis; Hyperdynamic; Portal hypertension; Splanchnic; Indocyanine green

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INTRODUCTION

We previously demonstrated that systemic hemodynamics

affecting postoperative graft function are crucial for living-donor liver transplantation (LDLT)[1]. However, the relationship between systemic hemodynamic parameters and splanchnic circulation after LDLT remains to be fully elucidated. In particular, the influence of the systemic hemodynamic state on splanchnic circulation is unclear. Therefore, we carried out a detailed investigation of systemic and splanchnic hemodynamic behavior after LDLT in adult recipients with cirrhosis.

Prior to undergoing LDLT, recipients with cirrhosis generally develop peculiar systemic and splanchnic hemodynamics due to portal hypertension[2-4]. To ascertain correlations between systemic hemodynamics and splanchnic circulation, and to clarify how the systemic hemodynamic state impacts on the local graft circulation, we performed simultaneous assessments of systemic hemodynamics and directly measured splanchnic circulation by systemic dye distribution and ultrasound. We also determined the hemodynamic state required for an excellent clinical outcome corresponding to good graft function.

MATERIALS AND METHODS

Patients

From June 2003 to March 2006, indocyanine green (ICG) pharmacokinetics were analyzed using a non-invasive method in 30 adult recipients (average age 53.1 ± 9.3 years; 25 males, five females) who underwent orthotopic LDLT at Mie University Hospital. As well, splanchnic circulatory parameters were simultaneously assessed using Doppler ultrasound. All 30 patients received a right-lobe liver graft. Clinical diagnoses were 26 cases of liver cirrhosis with hepatitis B or C (18 complicated by hepatocellular carcinoma), two cases of biliary atresia (result of postoperative state of Kasai's operation at childhood), and one case each of primary sclerosing cholangitis and alcoholic liver cirrhosis. All recipients were diagnosed with liver cirrhosis, based on histopathological examination of resected specimens. ABO blood group compatibility was identical in 24 recipients and compatible in six. The operative procedures and immunosuppression protocols used in our institute have been described in detail elsewhere[1,5-8]. All the protocols used in the present study were approved by the Ethics Review Committee for Human Studies of Mie University Graduate School of Medicine (Tsu, Mie, Japan), based on the Ethical Guidelines of the Helsinki Declaration of 1975. Informed consent was obtained from all patients before enrollment.

ICG, pulse dye densitometry (PDD) and analytical procedures

ICG is widely used for analysis of liver function[9,10]. Furthermore, the dye dilution curve of ICG can be used for measuring hemodynamic parameters[9,11]. A non-invasive method for measuring systemic hemodynamic parameters using ICG has been reported[12] and is relatively reliable compared with invasive ones[11,13-15]. It is also advantageous for clinical use because it is simple to use at the bedside, has quick real-time presentation of results and is cost-effective[16,17]. Hence, we used this non-invasive method in the present study.

ICG (Diagnogreen Inj., Daiichi Pharmaceutical, Tokyo, Japan), a non-toxic dye, has no known side effects other than a rare iodine allergy. Although a total of 630 ICG bolus injections were performed in the 30 recipients, no allergic responses or any other side effects were observed.

PDD, which measures the absorption of hemoglobin and ICG, is based on the principle of pulse spectrophotometry; the basic principles of which has been detailed elsewhere[11,12]. A PDD apparatus (DDG-2001; Nihon Kohden, Tokyo, Japan) was used to measure blood ICG concentrations and analyze dye densitography. A sensor was placed on the nose of each patient before ICG injection.

Twenty milligrams of ICG was injected through a peripheral cannula and immediately flushed with 20 mL normal saline[1,9,18]. PPD measurements were obtained before LDLT and from 1 to 14 d and at 21 d and 28 d postoperatively. In particular, measurements were performed every 12 h until 72 h postoperatively, because the hemodynamic parameters showed marked changes during the early postoperative period.

Systemic hemodynamic parameters and ICG elimination rate

The following parameters were measured and calculated using the PDD apparatus with the patients in a settled recumbent position: cardiac output (CO, L/min), cardiac index (CI, L/min per m^2), mean transit time (MTT, s, blood volume (BV, L), heart rate (HR, beats/min) and ICG elimination rate constant (K_{ICG}). MTT was defined as the time required for half the administered ICG to pass through the attached nasal sensor in the first circulation. Details of the above calculations have been described elsewhere[11,12,17]. Measurement of mean arterial pressure (MAP) was performed simultaneously with the PDD. MAP, calculated as MAP (mmHg) = (pulse pressure/3) + diastolic pressure, was measured using a standard manual method[19]. Total peripheral resistance (TPR) was subsequently calculated according to the following formula: TPR (dyne/s^5 per cm) = MAP × 80/CO[19].

Doppler ultrasound and splanchnic hemodynamic measurements

Doppler ultrasound assessment of splanchnic hemodynamic parameters was conducted at the same time as PDD. Portal venous flow velocity (PVFVe), portal venous flow volume (PVFVo), hepatic arterial pulsatility index (HAPI), and hepatic arterial resistance index (HARI) were evaluated as splanchnic circulatory parameters. A Triplex Doppler ultrasound system (Prosound SSD-5000SV; ALOKA, Tokyo, Japan) and a convex probe (2-5 MHz; UST-9119; ALOKA) were used for the Doppler ultrasound assessment. The following parameters were measured at the extrahepatic but post-anastomosis area: (1) PVFVe (cm/s), representing the mean of the maximal flow velocity of the portal vein; (2) PVFVo (mL/min), calculated from a cross-sectional area, assuming a circular portal vein section, and the mean velocity; (3) HAPI,

Table 1 Systemic hemodynamic parameters, K_{ICG} values and splanchnic circulatory parameters before LDLT

Parameters	Healthy individuals $n = 16$	Group Ⅰ $n = 25$	Group Ⅱ $n = 5$
Systemic hemodynamics			
CO (L/min)	5.83 ± 1.52	6.87 ± 0.97[a]	7.36 ± 1.07[e]
CI (L/min per m^2)	3.22 ± 0.71	4.10 ± 0.71[b]	4.56 ± 0.58[e]
BV (L)	3.40 ± 0.96	4.09 ± 0.51[a]	4.40 ± 0.45[e]
CO/BV (/min)	1.74 ± 0.28	1.69 ± 0.21	1.69 ± 0.28
MTT (s)	16.1 ± 2.3	16.5 ± 1.5	16.5 ± 1.2
HR (beat/min)	64.3 ± 9.9	77.9 ± 12.6[b]	77.6 ± 9.8[e]
MAP (mmHg)	89.3 ± 11.8	68.9 ± 6.5[d]	70.8 ± 11.2[e]
TPR (dyne/s^5 per cm)	1275.1 ± 228.3	818.9 ± 166.7[d]	785.3 ± 187.4[e]
ICG clearance test			
K_{ICG}	0.227 ± 0.076	0.037 ± 0.017[d]	0.056 ± 0.038[e]
Splanchnic circulation			
Portal vein			
PVFVo (mL/min)	1482.1 ± 335.6	327.3 ± 416.9[d]	435.6 ± 592.6[e]
PVFVe (cm/s)	45.1 ± 8.1	7.9 ± 12.8[d]	10.5 ± 13.8[f]
Hepatic artery			
HAPI	0.95 ± 0.11	1.06 ± 0.28[a]	1.16 ± 0.21[e]
HARI	0.93 ± 0.26	1.04 ± 0.23[a]	1.10 ± 0.10[e]

There were no significant differences between Groups I and Ⅱ in each parameter, respectively ($P > 0.05$, analyzed by Mann-Whitney's U test). Statistical differences between healthy individuals and Group I analyzed by Mann-Whitney's U test ([a]$P < 0.05$, [b]$P < 0.005$, [d]$P < 0.0005$). Statistical differences between healthy individuals and Group Ⅱ analyzed by Mann-Whitney's U test ([e]$P < 0.05$, [f]$P < 0.005$). ICG: Indocyanine green; LDLT: Living-donor liver transplantation; CO: Cardiac output; CI: Cardiac index; BV: Blood volume; MTT: Mean transit time; HR: Heart rate; MAP: Mean arterial pressure; TPR: Total peripheral resistance; PVFVo: Portal veinous flow volume; PVFVe: Portal venous flow velocity; HAPI: Hepatic arterial pulsatility index; HARI: Hepatic arterial resistant index.

calculated from the Doppler trace over one cardiac cycle as: (peak systolic velocity-minimum velocity)/mean of maximal velocities; and (4) HARI, derived from the Doppler spectrum over one cardiac cycle according to: (peak systolic velocity-end diastolic velocity)/peak systolic velocity. The measurement methods for the above indices have been described in detail elsewhere[20-23].

Establishment of normal ranges of systemic hemodynamic parameters, K_{ICG} value and splanchnic circulatory parameters

To establish the normal ranges of the variables we investigated the variables using the above-described methods in seven donors before LDLT and in nine volunteers who agreed to the aims of this study. The data measured in these 16 healthy individuals represent the normal ranges of the parameters, and are shown in Table 1. The control population showed no significant differences in age or body surface area compared with the LDLT recipients (data not shown).

Computed tomographic (CT) volumetry of liver grafts and the standard liver volume (SLV)

In our institution, helical CT studies are routinely performed at 2 and 4 wk after LDLT. All 30 recipients underwent these studies after LDLT. The helical CT studies were conducted using a High Speed Advantage QX-1 (GE Medical Systems, Tokyo, Japan). The scanning parameters were 120 kV, 200 mA, collimation of 5 mm, and a table speed of 15 mm/rotation, with reconstruction increments of 5 mm. Graft volume was calculated by CT volumetry. SLV was calculated according to a previously described formula[24].

Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl-human serum albumin (99mTc-GSA) liver scintigraphy and ratio of liver to heart-plus-liver radioactivity at 15 min (LHL15)

Since asialoglycoprotein receptors on hepatocytes are characteristic of functional liver cells[25], 99mTc-GSA liver scintigraphy is used as a reliable assessment tool for functional hepatic volume[26]. A total of 60 measurements were performed in the 30 recipients at 2 and 4 wk after LDLT. After intravenous injection of 185 MBq of 99mTc-GSA (Nihon Medi-Physics, Nishinomiya, Japan), dynamic imaging was performed with the patient in the supine position using a large field-of-view gamma camera (GCA7200A; Toshiba, Tokyo, Japan). LHL15 was calculated by dividing the radioactivity of whole liver regions of interest (ROIs) by that of whole liver-plus-heart ROIs at 15 min after injection, as previously described[27].

Histopathological analysis and graft parenchymal damage score

In our institution, needle biopsies are performed after LDLT if necessary. Protocol biopsies are not performed because of the associated risks, such as hemorrhage[28]. In the present study, a total of 30 biopsy specimens from the 30 recipients were assessed within 4 wk after LDLT.

Tissue specimens were stained with hematoxylin-eosin using standard histopathological techniques, and reviewed by an experienced liver pathologist using a semi-quantitative scoring system for features of the graft parenchyma. The graft parenchymal damage score, representing liver damage, was calculated as the total of the following parenchymal feature scores: hepatocyte ballooning (0, no; 1, yes), hepatocyte necrosis (0, none; 1, small foci; 2, confluent areas; 3, bridging necrosis), congestion (0, no; 1, yes), the fraction of hepatocytes that contain microvesicular fat (0, none; 1, < 1/3 of hepatocytes, 2, between 1/3 and 2/3 of hepatocytes; 3, > 2/3 of hepatocytes), neutrophil aggregates (0, none; 1, minimal; 2, moderate; 3, extensive) and cholestasis (0, none; 1, mild; 2, moderate; 3, severe). The graft parenchymal damage score, which was modified from the score according to Neil *et al*[29], has been described in detail elsewhere[6].

Outcomes after LDLT

The clinical courses of all recipients were followed for 996.2 ± 436.5 d, ranging from 32 (patient died) to 1472 d after LDLT. The 30 recipients were retrospectively divided into two groups based on clinical outcomes corresponding to postoperative graft function. Although 25 recipients (Group Ⅰ) presented with a good clinical course and excellent outcome, a subset of five recipients (Group Ⅱ) required long-term intensive management, and finally died because of hepatic or extrahepatic reasons that

Table 2 Clinical profiles before, during and after LDLT

Clinical profile	Group Ⅰ (n = 25)	Group Ⅱ (n = 5)	P value[a]
Before LDLT			
Age	51.8 ± 9.8	58.6 ± 3.5	NS
Body surface area (m^2)	1.69 ± 0.18	1.61 ± 0.12	NS
Child-Pugh score (points)	9.2 ± 2.3	10.8 ± 2.2	NS
Model for end-stage liver disease score (points)	17.6 ± 6.7	17.4 ± 7.1	NS
During LDLT			
Native liver weight (g)	857.0 ± 227.5	946.0 ± 376.2	NS
Portal venous pressure before removal of native liver (mmHg)	21.5 ± 4.7	24.6 ± 7.1	NS
Cold ischemic time (min)	163.7 ± 79.0	139.6 ± 52.6	NS
Warm ischemic time (min)	55.1 ± 16.7	45.8 ± 12.7	NS
Anhepatic phase (min)	209.2 ± 104.9	184.4 ± 177.6	NS
Operative time (min)	899.4 ± 126.7	933.4 ± 131.0	NS
Blood loss (mL)	22515.7 ± 14200.5	22788.6 ± 19247.8	NS
Graft weight (g)	687.8 ± 124.6	632.0 ± 72.9	NS
Graft-recipient weight ratio	1.09 ± 0.21	1.23 ± 0.37	NS
After LDLT			
Intensive care unit stay (d)	5.1 ± 1.9	35.6 ± 15.7	< 0.005
%SLV based on CT volumetry			
2 wk after LDLT	1.14 ± 0.22	1.07 ± 0.09	NS
4 wk after LDLT	1.05 ± 0.15	1.17 ± 0.16	NS
LHL15 based on ^{99m}Tc-GSA liver scintigraphy			
2 wk after LDLT	0.935 ± 0.026	0.846 ± 0.061	< 0.005
4 wk after LDLT	0.941 ± 0.017	0.751 ± 0.034	< 0.005
Histopathological fraft parenchymal damage score (points)			
Within 4 wk after LDLT	3.9 ± 1.4	10.6 ± 1.3	< 0.005

Statistical differences between Groups I and Ⅱ analyzed by Mann-Whitney's U test (NS: $P > 0.05$). LDLT: Living-donor liver transplantation; SLV: Standard liver volume; CT: Computed tomographic; LHL15: The ratio of liver to heart-plus-liver radioactivity at 15 min; 99mTc-GSA: Technetium-99m-diethylenetriaminepenta-acetic acid-galactosyl-human serum albumin.

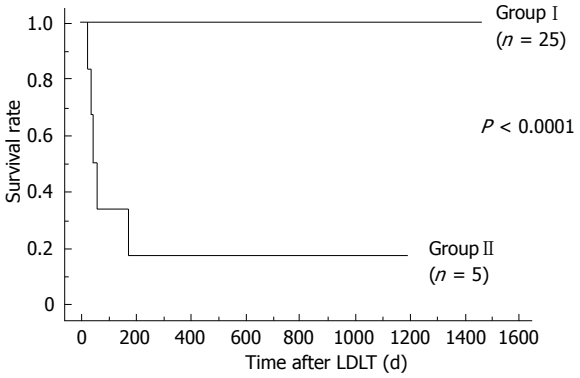

Figure 1 Survival rates after LDLT. The two lines represent the survival rates for Groups Ⅰ and Ⅱ. The P value analyzed by the log-rank test was < 0.0001.

originated from graft dysfunction with prolonged jaundice. Group Ⅱ showed poor clinical outcome, and survival rate differed significantly between the two groups ($P < 0.0001$) (Figure 1).

Clinical profiles before, during and after LDLT

There were no significant differences in the clinical profiles before and during LDLT between the two groups. We considered that high portal venous pressure before removal of the native liver was due to portal hypertension. After LDLT, there was a significant difference in the length of stay in the intensive care unit between the two groups. Although there were no significant differences in SLV, LHL15 and graft parenchymal damage scores both differed significantly between the two groups (Table 2). Because LHL15 and graft parenchymal damage scores accurately reflect functional hepatocytes, these results clearly indicated graft dysfunction in Group Ⅱ during the late postoperative period after LDLT.

Statistical analysis

Results were expressed as means ± SD. For individually, temporally and repeatedly measured data, differences in the changes over time after LDLT between the two groups were analyzed by repeated-measures ANOVA[30,31]. Differences in unpaired discontinuous data between the two groups were analyzed by Mann-Whitney's U test. Survival rates were calculated by the Kaplan-Meier method, and the log-rank test was used for between-group comparisons of recipient survival. All calculations were performed using Stat View-J 5.0 statistical software (SAS Institute, Cary, NC, USA) and values of $P < 0.05$ were considered significant.

RESULTS

Systemic hemodynamic states before LDLT and temporal differences in systemic hemodynamic parameters after LDLT

Cirrhotic systemic hemodynamics have been symbolized as hyperdynamic[1-4,32], and the hyperdynamic state characterized by high CO or CI, large BV, low TPR, mild tachycardia, and low or normal MAP[2,19,32-35]. Although hyperdynamic states were recognized in both groups, there were no significant differences between the two groups before LDLT. Interestingly, CO/BV and MTT were both constant before LDLT (Table 1). There were significant

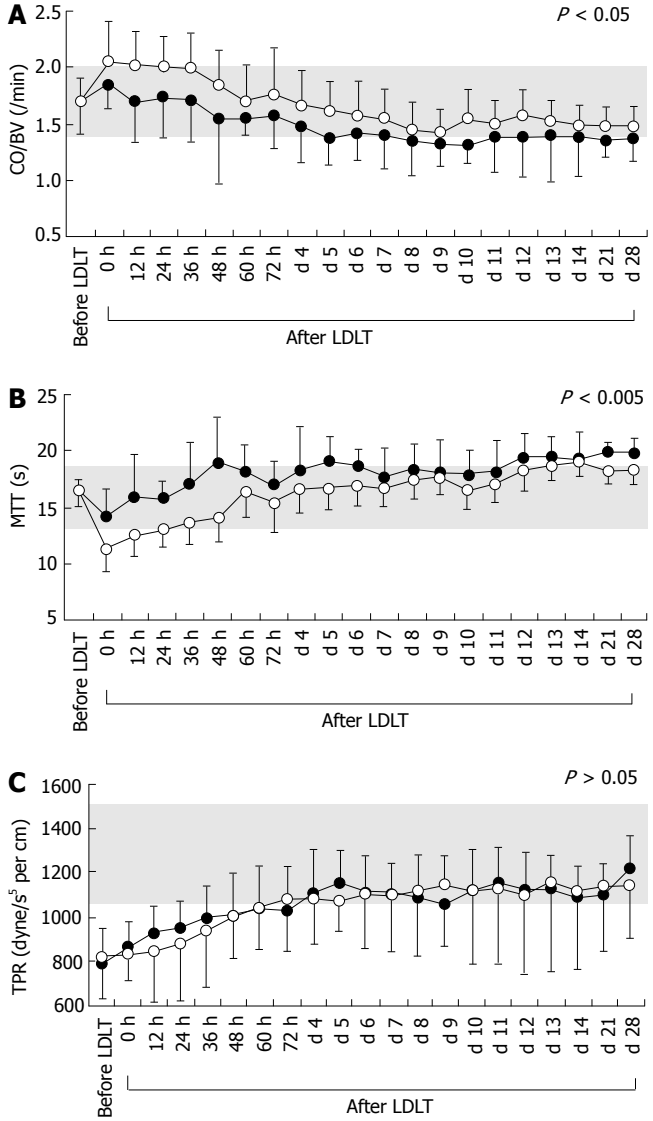

Figure 2 Temporal changes in systemic hemodynamic parameters before and after LDLT. **A:** Temporal changes in the ratio of CO to BV before and after LDLT; **B**: Temporal changes in MTT before and after LDLT; **C**: Temporal changes in TPR before and after LDLT. Open and closed circles represent systemic hemodynamic parameters for Groups Ⅰ and Ⅱ, respectively. Shaded areas show normal ranges measured in healthy individuals.

temporal differences after LDLT between the groups for CO/BV and MTT, but no significant differences in CO, CI, BV, HR, MAP or TPR (Table 3). The actual temporal changes in CO/BV, MTT and TPR are presented in Figure 2. When the absolute values of CO and BV in the recipients were compared with those of healthy individuals, recipients in Group Ⅰ persisted in a hyperdynamic state after LDLT, while those in Group Ⅱ showed a tendency to remain in a hyperdynamic state (actual temporal changes not shown). Thus, regardless of the outcome and graft function, the temporal changes in the absolute values of CO and BV between groups did not reach statistical significance. Therefore, as we have previously determined, detecting subtle disorders of optimal systemic hemodynamics in recipients with cirrhosis by comparing absolute values is not necessarily satisfactory (unpublished data). Indicators for peripheral resistance are thought to precisely reflect cirrhotic vascular alterations and the

Table 3 Statistical differences in post-operative temporal changes of systemic hemodynamic parameters, K_{ICG} values and splanchnic circulatory parameters

Parameters	Statistical temporal differences after LDLT between Groups Ⅰ and Ⅱ *P* value[1]
Systemic hemodynamics	
CO (L/min)	0.2321
CI (L/min per m^2)	0.5037
BV (L)	0.3420
CO/BV (/min)	0.0426[a]
MTT (s)	0.0023[b]
HR (beat/min)	0.0701
MAP (mmHg)	0.2453
TPR (dyne/s^5 per cm)	0.8859
ICG clearance test	
K_{ICG}	0.0001[d]
Splanchnic circulation	
Portal vein	
PVFVo (mL/min)	0.0113[a]
PVFVe (cm/s)	0.0171[a]
Hepatic artery	
HAPI	0.2504
HARI	0.4261

[1]Statistical temporal differences between Groups I and Ⅱ analyzed by repeated measures ANOVA ([a]$P < 0.05$, [b]$P < 0.005$, [d]$P < 0.0005$). ICG: Indocyanine green; LDLT: Living-donor liver transplantation; CO: Cardiac output; CI: Cardiac index; BV: Blood volume; MTT: Mean transit time; HR: Heart rate; MAP: Mean arterial pressure; TPR: Total peripheral resistance; PVFVo: Portal veinous flow volume; PVFVe: Portal venous flow velocity; HAPI: Hepatic arterial pulsatility index; HARI: Hepatic arterial resistant index.

presence of collateral vessels and shunts[19,36,37]. It should be noted that the changes in TPR in the two groups exhibited similar patterns with no prompt restoration, despite normalization of the portal pressure after LDLT, and showed quite slow improvement (Figure 2C).

K_{ICG} before LDLT and differences in temporal changes in K_{ICG} after LDLT

Recipients in both groups showed large decreases in K_{ICG} before LDLT (Table 1). Although there were no significant differences in K_{ICG} between the groups before LDLT, K_{ICG} changed significantly after LDLT (Figure 3, Table 3). The K_{ICG} value is dualistic, since it reflects functional hepatocytes and splanchnic blood flow[9,38-40]. However, splanchnic blood flow is a major determinant of K_{ICG} in normal liver[9,41,42]. We have previously demonstrated that K_{ICG} accurately evaluates functional hepatocytes during the late postoperative period, and sharply reflects splanchnic circulation during the early postoperative period, since LDLT restores functional hepatocyte volume drastically and immediately[1]. Extraordinary decreases in K_{ICG} from the early postoperative period were observed in Group Ⅱ, in contrast to the findings for Group I. Therefore, in the present study we verified the detailed splanchnic circulatory parameters measured by Doppler ultrasound.

Splanchnic hemodynamics before LDLT and temporal differences in splanchnic circulatory parameters after LDLT

Cirrhotic splanchnic circulation is symbolized by decreased

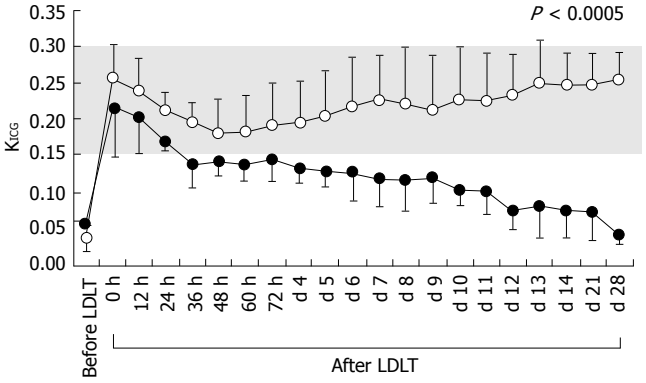

Figure 3 Temporal changes in K_{ICG} before and after LDLT. Open and closed circles represent K_{ICG} values for Groups Ⅰ and Ⅱ, respectively. The shaded area shows the normal range measured in healthy individuals.

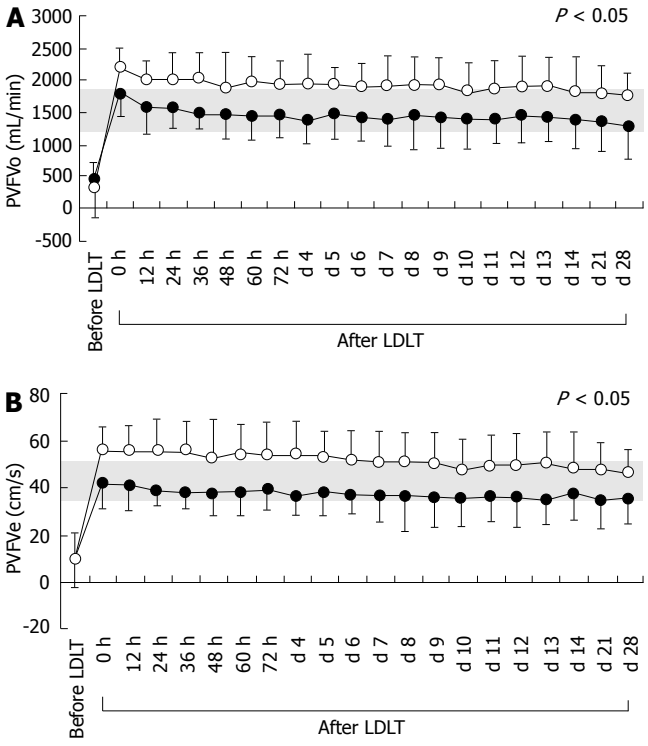

Figure 4 Temporal changes in splanchnic circulatory parameters before and after LDLT. **A:** Temporal changes in PVFVo before and after LDLT; **B**: Temporal changes in PVFVe before and after LDLT. Open and closed circles represent splanchnic circulatory parameters for Groups Ⅰ and Ⅱ, respectively. Shaded areas show normal ranges measured in healthy individuals.

portal venous flow because of portal hypertension, despite a systemic hyperdynamic state. Although all splanchnic circulatory parameters in both groups before LDLT differed significantly from those in healthy individuals, there were no significant differences between the two groups (Table 1). However, after LDLT, there were significant temporal differences in PVFVo and PVFVe, but no significant differences in HAPI and HARI, between the two groups (Table 3). The actual temporal changes in PVFVo and PVFVe are shown in Figure 4. Interestingly, differences in portal venous parameters, but not hepatic arterial parameters, were observed.

DISCUSSION

Almost all adult recipients who undergo LDLT develop liver cirrhosis with long-term portal hypertension. Portal hypertension results in vascular dilatation and collateral pathways. Thus, various alterations in systemic hemodynamics and splanchnic circulation occur, and adult recipients often present characteristic hemodynamics before LDLT. Cirrhotic hemodynamic abnormalities were obviously present before LDLT in the present study.

Several investigators have demonstrated that the systemic hyperdynamic state remains despite normalization of liver function and restoration of portal pressure after LDLT[19,33,36,43-45], and have suggested that most systemic parameters are slowly restored to the normal range after LDLT[19,36]. In agreement with these suggestions, our results demonstrated that vascular alterations do not disappear within 4 wk after LDLT, regardless of the outcome. Thus, we have suggested that optimal persistence of a systemic hyperdynamic state after LDLT is necessary for successful outcomes in recipients with cirrhosis (unpublished data).

A cirrhotic systemic hyperdynamic state is symbolized by expanded BV, high CO and low TPR[3,9,32], and the preload focuses on the balance between CO and BV[46,47]. Thus, we suggest that the balance of CO to BV is an accurate estimator of the optimal stability of the characteristic systemic hyperdynamic state (unpublished data). On the other hand, to determine the systemic hemodynamic parameters related to liver transplantation, the MTT is a rigorous indicator of kinetic behavior circuits[1,9]. MTT values precisely reflect systemic hemodynamics, which are especially influenced by preload factors. That is, a greater CO is proportional to a shorter MTT, and a large BV is proportional to a prolonged MTT. Accordingly, CO/BV and MTT represent mirror images. The results presented here showed significant temporal differences between the two groups in these precise systemic hemodynamic parameters. Thus, we suggest that the recipients in Group Ⅱ showed subtle disorders of the systemic hyperdynamic state after LDLT, in contrast to the recipients in Group Ⅰ.

Other studies have focused on systemic hemodynamics or splanchnic circulation after LDLT, and some investigators have demonstrated that systemic hemodynamics are well correlated with the splanchnic circulation[41,44,48]. Interestingly, the results for the splanchnic circulatory parameters in the current study reveal that subtle disorders of the optimal systemic hyperdynamic state easily influence portal venous flow, rather than hepatic arterial flow. Vascular alterations because of portal hypertension develop in vessels that originally flow into the portal vein under normal portal pressure, and represent one of the causes of a large BV. Hence, we suggest that the imbalance between the greater CO and larger BV after LDLT in Group Ⅱ caused stagnation of the tributary blood flow in the dilated vein and collateral pathways, which resulted in a decrease in portal venous flow. It was also of interest that recipients with cirrhosis with good

outcomes (i.e., Group I) showed a clear tendency toward postoperative portal venous overflow compared with that in healthy individuals. We have previously demonstrated that the persistence of a systemic hyperdynamic state is indispensable for recipients with cirrhosis after LDLT (unpublished data), and therefore consider that excessive portal flow after LDLT seems to be correlated with a postoperative systemic hyperdynamic state. Since portal venous flow has been shown to have a large influence on liver regeneration after LDLT[49], we conclude that successful clinical outcomes in cirrhotic LDLT recipients can be attributed to optimal stability of the systemic hyperdynamic state, which yields sufficient portal venous flow. Based on our results for Group I as compared with Group Ⅱ, we suggest that continuous sufficient portal venous flow, with even a slight surplus, supported by the optimal systemic hyperdynamic state, is necessary for good outcomes after LDLT in recipients with cirrhosis. Since reversible graft damage might begin slowly from the early postoperative period, we suggest that appropriate intensive clinical management of hemodynamics will greatly impact on further improvements in LDLT outcomes.

COMMENTS

Background

Prior to undergoing LDLT, recipients with cirrhosis generally develop peculiar systemic and splanchnic hemodynamics due to portal hypertension. To ascertain correlations between systemic hemodynamics and splanchnic circulation, we performed simultaneous assessment of systemic hemodynamics and directly measured splanchnic circulation by systemic dye distribution and ultrasound.

Research frontiers

We clarify how the systemic hemodynamic state impacts on the local graft circulation in recipients with cirrhosis who underwent LDLT. Vascular alterations due to portal hypertension develop in vessels that originally flow into the portal vein under normal portal pressure, and represent one of the causes of a large BV. Hence, we suggest that the imbalance between the greater CO and larger BV after LDLT caused stagnation of the tributary blood flow in the dilated veins and collateral pathways, which resulted in a decrease in portal venous flow.

Innovations and breakthroughs

We also identified the hemodynamic state required for an excellent clinical outcome after LDLT. Since portal venous flow has been shown to have a large influence on liver regeneration after LDLT, we suggest that successful clinical outcomes in LDLT recipients with cirrhosis can be attributed to optimal stability of the systemic hyperdynamic state, which yields sufficient portal venous flow.

Applications

The methods in this study (PDD and ultrasound) are advantageous for clinical applications because of their simplicity of bedside use, rapid real-time presentation of results, and cost-effectiveness. Hence, we suggest that appropriate intensive clinical management of hemodynamics based on real-time and reliable results measured by non-invasive methods will have a large impact on further improvements in LDLT outcomes.

Terminology

Splanchnic blood flow in this study refers to that in cirrhotic recipients after living-donor liver transplantation.

Peer review

This study builds on previous observations by the same group that hyperdynamic systemic circulation persists following transplantation in patients who previously had cirrhosis, and that this is important for sustaining portal venous flow. The current manuscript focuses on the changes with respect to splanchnic hemodynamics. The authors have demonstrated significant differences in portal venous flow dynamics between a group of 25 individuals that had a good clinical outcome post-transplantation compared with five that had a poor postoperative course. This article has sufficient originality regarding the understanding of post-liver transplant hemodynamics.

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CLINICAL RESEARCH

Expression of matrix metalloproteinase-1 and tumor necrosis factor-α in ulcerative colitis

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Abstract

AIM: To examine the expression of matrix metalloproteinase-1 (MMP-1) and tumor necrosis factor-α (TNF-α) in the colon mucosa of patients with ulcerative colitis (UC).

METHODS: Reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry were used to examine the expression of MMP-1 and TNF-α at both mRNA and protein levels in the colon mucosa of patients with UC. Correlation between MMP-1 and TNF-α and their correlation with the severity of the disease were also analyzed statistically.

RESULTS: The expression of MMP-1 and TNF-α in the ulcerated and inflamed colon mucosa of patients with UC was significantly higher than that in the non-inflamed mucosa of normal controls at both mRNA and protein levels. Furthermore, the expression of MMP-1 and TNF-α in the ulcerated area was significantly higher than that in the inflamed area of patients with UC (0.9797 ± 0.1433 *vs* 0.6746 ± 0.0373, 0.8669 ± 0.0746 *vs* 0.5227 ± 0.0435, $P < 0.05$). There was no statistically significant difference in the non-inflamed area of normal controls. There was a significant correlation between MMP-1 and TNF-α expression (0.9797 ± 0.1433 *vs* 0.8669 ± 0.0746, $P < 0.05$), the correlating factor was 0.877. MMP-1 and TNF-α showed a significant correlation with the severity of the disease (0.0915 ± 0.0044 *vs* 0.0749 ± 0.0032 , 0.0932 ± 0.0019 *vs* 0.0724 ± 0.0043, $P < 0.05$), their correlating factors were 0.942 and 0.890, respectively.

CONCLUSION: Excessively expressed MMP-1 directly damages the colon mucosa by degrading extracellular matrix (ECM) in patients with UC. While damaging colon mucosa, excessively expressed TNF-α stimulates MMPs secreting cells to produce more MMP-1 and aggravates the mucosa damage. MMP-1 promotes secretion of TNF-α in a positive feedback manner to cause further injury in the colon mucosa. MMP-1 and TNF-α correlate well with the severity of the disease, and therefore, can be used clinically as biological markers to judge the severity of UC.

Key words: Ulcerative colitis; Matrix metalloproteinase-1; Tumor necrosis factor-α; Reverse transcription-polymerase chain reaction; Immunohistochemistry

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease of the colon mucosa with an increasing morbidity due to life pattern changes in China. However, its etiology and pathogenesis are still unknown. Pathophysiologically, ulceration in the mucosal and submucosal areas of patients with UC is due to excessive degradation of extracellular matrix (ECM). In recent years, matrix metalloproteinases (MMPs) and some cytokines have been implicated in the development of a number of diseases, such as multiple sclerosis, rheumatic disease and UC[1-3]. In patients with UC, MMPs participate in tissue repair, vascularization and leucocyte chemotaxis in the ulcerated and inflamed colonic mucosa[3]. MMP-1 produced by cytokine-activated interstitial cells is one of the most important enzymes in degrading ECM[4]. Excessive expression of MMP-1 in the diseased colon mucosa of UC patients causes excessive hydrolysis of the ECM and ulceration[5,6]. It is also believed that imbalance between inflammatory and anti-inflammatory cytokines plays a central role in the development of UC[7]. For example, TNF-α, an important inflammatory cytokine produced by macrophages in the colon, takes part in the pathogenesis of UC[8] and can directly damage the colonic mucosal barrier, causing inflammatory changes in UC. Therefore, in this study we measured MMP-1 and TNF-α transcript and their proteins using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry to explore

their possible role and interrelationship in the pathogenesis of UC.

MATERIALS AND METHODS

Patients and samples

Thirty-six patients with UC confirmed by clinical manifestations, colonoscopy and biopsy were enrolled in this study. Among these patients, 15 were males and 21 were females with their age ranged from 22 to 72 years and averaged 44 years. Samples were taken from the ulcerated, inflamed and non-inflamed areas of the colon mucosa during colonoscopy. There were 4 patients with pan-colon lesions, 3 with hemi-colon lesions, 19 with recto-sigmoid lesions, and 10 with rectal lesions. Based on the clinical manifestations and colonoscopic findings, 8 patients were classified into mild type, 21 into moderate type, and 7 into severe type. Meanwhile, 20 normal subjects were chosen as normal controls, 12 of them were males and 8 were females with their age ranged from 22 to 56 years and averaged 34 years. Biopsy samples were immediately snap frozen in liquid nitrogen and stored at -80℃ for RT-PCR. Biopsy samples were fixed in formalin, embedded in paraffin and cut into 4 μm-thick sections for immunohistochemistry.

Total RNA extraction

Total RNA was extracted from the frozen samples using a RNA isolation kit (Invitrogen Company) following the manufacturer's instructions. Five μL of the extracted RNA was run on 1% agarose gel electrophoresis to identify the extracted products.

RT-PCR for MMP-1 and TNF-α

RT-PCR was performed using the TaKaRa RNA PCR kit 3.0 (AMV) (supplied by Dalian Baosheng Biotechnology Company) following the manufacturer's instructions. Primer sequences used are as follows: MMP-1 (sense: 5'-ATGCGAACAAATCCCTTCTACC-3', antisense: 5'-TTCCTCAGAAAGAGCAGCATCG-3'), TNF-α: (sense: 5'-CTGTAGCCCATGTTGTAGC-3', antisense: 5'-CAATGATCCCAAAGTAGACCT-3'). Primers for β-actin were used as the internal control (sense: 5'-CCTTCCTGGGCATGGAGTCCTG-3', antisense: 5'-GGAGCAATGATCTTGATCTTC-3'). Reverse transcription was carried out at 30℃ for 10 min, at 42℃ for 30 min, at 99℃ for 5 min, and at 5℃ for 5 min. PCR was performed as follows: initial denaturation at 94℃ for 2 min, followed by 35 amplification cycles at 94℃ for 30 s, at 53℃ for 30 s, at 72℃ for 1 min, extension at 72℃ for 10 min. Five μL of PCR products was run on 2% agarose gel electrophoresis.

Immunohistochemistry

Sample sections were washed 3 times with PBS, 3 min each time after initial treatment. Primary antibodies, mouse anti-human MMP-1 monoclonal antibody and rabbit anti-human TNF-α polyclonal antibody (Beijing Zhongshan Biology Company) were added and incubated at room temperature for 1.5 h, washed again and incubated with peroxidase-conjugated secondary antibody for 15 min and washed again. A brown product was developed in diaminobenzidine (DAB) for 10 min.

Result determination and statistical analysis

A bio-imaging system (PALL Company, USA) was employed to analyze the density of the bands of PCR products. MMP-1 mRNA and TNF-α mRNA were semi-quantitatively expressed by the ratios between MMP-1, TNF-α and β-actin OD values. All values were expressed as mean ± SD.

Results of immunohistochemistry were considered positive when brown particles appeared in the cells after DAB staining. An image-pro-plus 4.5 microscopic image analyzing system was used to measure the density of the positive products. Five fields in each section were randomly selected to measure the total density and area. The mean density was determined by calculating the ratio between the total density and area in each section. A bigger ratio value indicates a greater expression of the corresponding proteins.

Student-Neuman-Keuls test was used to compare MMP-1 and TNF-α: mRNAs and their corresponding proteins in different colon samples and in different severity of the disease. Spearman correlation analysis was used to study the relationship between MMP-1, TNF-α and severity of the disease. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for windows.

RESULTS

Expression of MMP-1 and TNF-α mRNA in different colon areas of UC patients

The expression of MMP-1 and TNF-α mRNA in the ulcerated area of colon was significantly higher than that in the inflamed colon area of patients with UC and non-inflamed colon area of normal controls ($P < 0.05$). The expression of MMP-1 and TNF-α mRNA in the inflamed colon area of patients with UC was also significantly higher than that in the non-inflamed colon area of normal controls ($P < 0.05$), but the extent was not as high as that in the ulcerated area. There was no statistically significant difference in non-inflamed colon area of normal controls (Table 1, Figures 1 and 2).

Expression of MMP-1 and TNF-α mRNA in patients with different severity of UC

The expression of MMP-1 mRNA was significantly higher in different groups of patients than in normal controls ($P < 0.05$). Comparison among the three groups showed that the highest expression of MMP-1 and TNF-α mRNA was seen in the group of patients with severe UC followed by in groups of patients with mild and moderate UC (Table 2).

Correlation MMP-1 and TNF-α mRNA expression

Correlation studies showed that the expression of MMP-1 mRNA was significantly correlated with that of TNF-α mRNA. The correlating factor was 0.877 ($P < 0.01$).

Table 1 Expression of MMP-1 and TNF-α mRNA in samples from different areas of colon of UC patients (mean ± SD)

Samples	MMP-1 mRNA	TNF-α mRNA	*P* value
Ulcerated area	0.9797 ± 0.1433	0.8669 ± 0.0746	< 0.05[a,c,e]
Inflamed area	0.6746 ± 0.0373	0.5227 ± 0.0435	< 0.05[a,c]
Non-inflamed area	0.0071 ± 0.0025	0.0302 ± 0.0299	> 0.05
Normal controls	0.0062 ± 0.0029	0.0280 ± 0.0060	

[a]*P* < 0.05 *vs* non-inlamed area; [c]*P* < 0.05 *vs* normal controls; [e]*P* < 0.05 *vs* inflamed area.

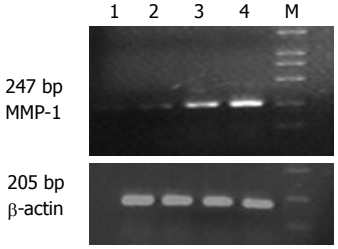

Figure 1 MMP-1 mRNA RT-PCR. Lane 1: Normal controls; lane 2: Non-inflamed area; lane 3: Inflamed area; lane 4: Ulcerated area; lane M: Marker.

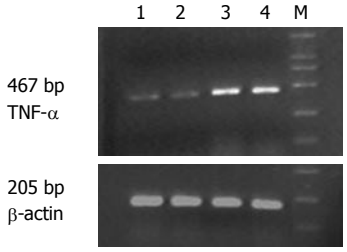

Figure 2 TNF-α mRNA RT-PCR. Lane 1: Normal controls; lane 2: Non-inflamed area; lane 3: Inflamed area; lane 4: Ulcerated area; lane M: Marker..

The expression of MMP-1 and TNF-α mRNA was also significantly correlated with the severity of the disease. The correlating factor was 0.942 and 0.890, respectively ($P < 0.01$).

Results of immunohistochemistry

Immunohistochemistry showed that the expression of MMP-1 and TNF-α in different areas of colon was identical. The expression of MMP-1 and TNF-α in the ulcerated area was significantly higher than that in the inflamed colon area of UC patients and non-inflamed colon area of normal controls ($P < 0.05$). The expression of MMP-1 and TNF-α in the inflamed colon area of UC patients was also significantly higher than that in the non-inflamed colon area of normal controls ($P < 0.05$), but it was not as high as that in the ulcerated area. There was no statistically significant difference in the non-inflamed colon area of normal controls (Figure 3A-D and Figure 4A-D, Table 3).

Protein analysis showed that the expression of MMP-1 and TNF-α in patients with different severity of the disease was identical The expression of MMP-1 and TNF-α was significantly higher in different groups than that in normal controls ($P < 0.05$). Comparison among the three groups showed that the highest expression of MMP-1 and TNF-α was seen in the group of patients with severe UC followed by in groups with mild and moderate UC (Table 4).

Table 2 Expression of MMP-1 mRNA in samples from patients with different severity of UC (mean ± SD)

Samples	MMP-1 mRNA	TNF-α mRNA	*P* value
Ulcerated area	0.9797 ± 0.1433	0.8669 ± 0.0746	< 0.05[ace]
Inflamed area	0.6746 ± 0.0373	0.5227 ± 0.0435	< 0.05[ac]
Non-inflamed area	0.0071 ± 0.0025	0.0302 ± 0.0299	< 0.05
Normal controls	0.0062 ± 0.0029	0.0280 ± 0.0060	

[a]*P* < 0.05 *vs* moderate type; [c]*P* < 0.05 *vs* severe type; [e]*P* < 0.05 *vs* normal controls.

Table 3 Expression of MMP-1 and TNF-α proteins in samples from different areas of colon of UC patients (mean ± SD)

Samples	MMP-1	TNF-α	*P* value
Ulcerated area	0.0891 ± 0.0062	0.0903 ± 0.0054	< 0.05[a,c,e]
Inflamed area	0.0791 ± 0.0047	0.0832 ± 0.0028	< 0.05[a,c]
Non-inflamed area	0.0047 ± 0.0040	0.0036 ± 0.0013	> 0.05
Normal controls	0.0048 ± 0.0016	0.0029 ± 0.0021	

[a]*P* < 0.05 *vs* non-inlamed area; [c]*P* < 0.05 *vs* normal controls; [e]*P* < 0.05 *vs* inflamed area.

Table 4 Expression of MMP-1 and TNF-α protein in samples from UC patients with different severity of the disease (mean ± SD)

Samples	MMP-1	TNF-α	*P* value
Mild type	0.0749 ± 0.0032	0.0724 ± 0.0043	< 0.05[a,c,e]
Moderate type	0.0812 ± 0.0030	0.0840 ± 0.0036	< 0.05[a,e]
Severe type	0.0915 ± 0.0044	0.0932 ± 0.0019	< 0.05[e]
Normal controls	0.0048 ± 0.0016	0.0029 ± 0.0021	

[a]*P* < 0.05 *vs* moderate type; [c]*P* < 0.05 *vs* severe type; [e]*P* < 0.05 *vs* normal controls.

DISCUSSION

Ulcerative colitis (UC) is a chronic, non-specific inflammatory disease with ulceration in the mucosal and submucosal areas of colon. Excessive degradation and insufficient synthesis of extracellular matrix (ECM) are the main pathophysiological events occurring in the process of ulceration. Since matrix metalloproteinases (MMPs) are the major hydrolytic enzymes that degrade ECM, the increased activity of MMPs is responsible for tissue damage of the colon in UC patients. It has been well accepted that inflammatory cytokines including TNF-α participate in the pathogenesis of UC[7]. The relationship between MMPs and inflammatory cytokines remains to be studied when both of them take part in the pathogenesis of UC.

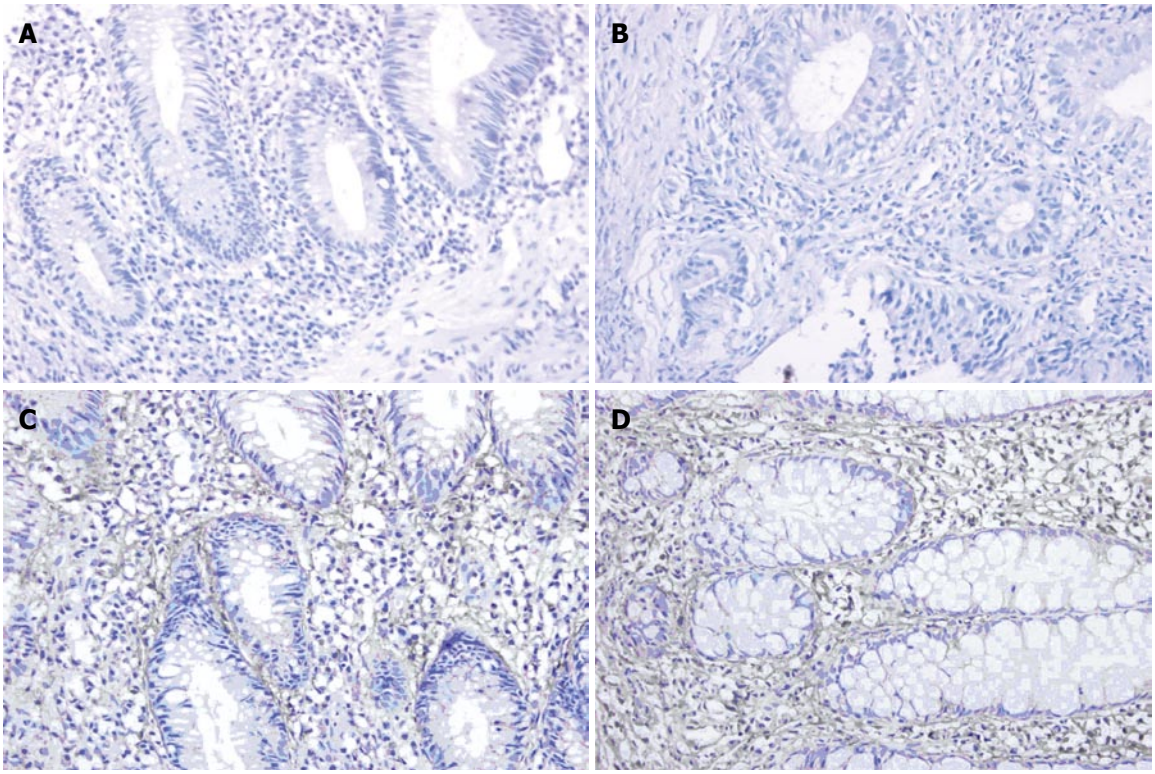

Figure 3 Expression of MMP-1 in normal controls (**A**), in non-inflamed area (**B**), in inflamed area (**C**), and in ulcerated area (**D**).

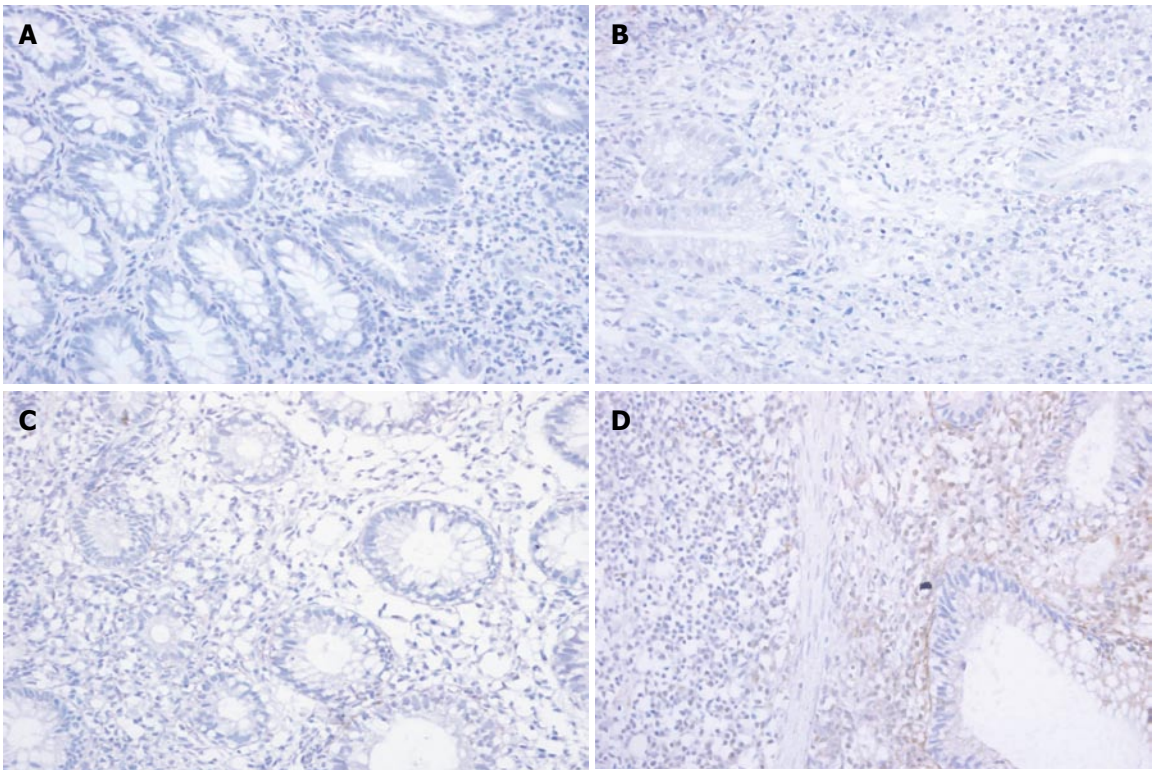

Figure 4 Expression of TNF-α in normal controls (**A**), in non-inflamed area (**B**), in inflamed area (**C**), and in ulcerated area (**D**).

MMPs are a group of zinc-dependent peptidases that degrade ECM. MMP-1, also known as interstitial collagenase, degrades mainly collagen types Ⅰ, Ⅱ, Ⅲ, Ⅵ, Ⅸ and proteoglycan, and plays an important role in degrading ECM in UC patients. Using RT-PCR and immunohistochemistry, we found that at both transcription and protein levels, the expression of MMP-1 in ulcerated and inflamed colon area of patients with UC

was significantly higher than that in non-inflamed colon area of normal controls. Furthermore, the expression of MMP-1 in ulcerated area was significantly higher than that in the inflamed area. In the present study, MMP-1 expression was closely correlated with the severity of the disease (correlating factor was 0.942, $P < 0.05$), indicating that MMP-1 is closely related to colon mucosa damage in UC patients[9,10]. Immunohistochemistry showed that MMP-1 was expressed mainly in the cytoplasm of mono-macrophages, which is consistent with the results reported by Von Lampec *et al*[11]. McKaig *et al*[12] also found that MMP-1 is expressed in damaged tissue vascular smooth muscle cells, indicating that MMP-1 may be related with formation of new blood vessels.

Our results showed that at transcription and protein levels, the expression of TNF-α in the ulcerated and inflamed area of UC patients was significantly higher than that in the non-inflamed area of normal controls. The expression of TNF-α was closely correlated with the severity of the disease (correlating factor was 0.890, $P < 0.05$), indicating that the more severe the disease, the higher the TNF-α expression. Immunohistochemistry revealed that the TNF-α positively stained cells were mainly mono-macrophages. Ishiguro[13] also reported that TNF-α expression in the diseased mucosa of colon in UC patients is significantly higher than that in the unaffected area of normal controls, suggesting that lipopolysaccharide produced by the intestinal flora may directly activate macrophages in the lamina propria, proliferating and producing a series of cytokines including TNF-α which damage the mucosa barrier of colon and produce typical inflammatory changes in UC. Apart from inflammatory cytokines, anti-inflammatory cytokines such as IL-10 also take part in the pathogenesis of UC. Gasche *et al*[14] reported that the expression of IL-10 mRNA is significantly decreased while Niessner *et al*[15] found that IL-10 mRNA is highly expressed in active UC, indicating that the expression of IL-10 mRNA is different in UC patients. Using in situ hybridization and immunohistochemical methods, Autschbach *et al*[16] showed that the number of IL-10 secreting monocytes and the mucosal expression of IL-10 are both significantly increased, but the expression of IL-10 in lanmina propria is relatively low, suggesting that IL-10 cannot effectively inhibit inflammatory cytokines such as TNF-α in lamina propria.

In the present study, MMP-1 was found to be closely correlated with TNF-α, indicating that there is a certain relationship between MMPs and cytokines. There is evidence that multiple cytokines may influence the expression of MMPs during inflammatory responses. Previous studies indicate that IL-1β and TNF-α are potent stimulators of MMP-1 and MMP-3[17,18]. They can regulate the secretion of MMP-1 and MMP-3 produced by mono-macrophages. Sylvia *et al*[19] found that the activity of T cells is correlated with the extent of colon mucosa damage, and that the colon mucosa injury is mediated by endogenously produced MMPs. Some authors believe that anti-inflammatory cytokines, such as IL-4 and IL-10, are able to inhibit the secretion of MMPs by monocytes[20-22]. Qiu *et al*[23] found that MMP-2 and MMP-9 combine with CD44 receptors on the cell membrane to form MMP-1/19-CD44 complex, making the inactivated TGF-β become its active form through hydrolysis and carry out its biological functions. Black *et al*[24] reported that MMPs activate TNF-α on cell membrane through hydrolysis to make it in an active state. MMPs may also block some cytokines, such as IL-6 and TGF-α to down-regulate their activities[25]. It is believed that MMPs not only appear in the down stream of inflammatory responses but also exert a positive feedback effect on cytokines. Therefore, they can be regarded as important "regulators" of inflammatory responses.

MMPs and cytokines play an important role in the process of UC. When infection, diet or other environmental factors act on hereditarily susceptible individuals, abnormal immune responses of the intestine may activate immune cells (such as T cells, lymphocytes and macrophages) to secrete a big amount of cytokines, inflammatory mediators and complements. These substances directly damage the colon mucosa, and induce interstitial cells (including smooth muscle cells, fibroblasts and mono-macrophages) to secrete MMPs. The increased MMPs degrade ECM in the colon mucosa, leading to mucosa damage and ulceration. While cytokines influence MMPs expression, and MMPs themselves are able to up-regulate cytokines through certain ways to cause further damage on the colon mucosa, MMPs can be inhibited by their inhibitors (MMPI) including their natural ones[26], revealing that MMPs have become one of the targets in anti-inflammatory treatment. MMPs inhibitors used in treatment of malignant tumors in clinical phase Ⅲ trial[27] in America and Europe can also be used in the treatment of patients with UC[7], while anti-inflammatory or inflammatory cytokine inhibitors can be used to reduce MMPs expression so as to indirectly reduce tissue damage and ulceration in UC patients. For example, a TNF-α antagonist, Infliximab, has been proved effective against adult and children UC patients[28,29].

In conclusion, excessively expressed MMP-1 directly damages the colon mucosa by degrading ECM in UC patients. While damaging colon mucosa, excessively expressed TNF-α stimulates MMPs secreting cells to produce more MMP-1 and aggravates the mucosa damage. MMP-1 promotes secretion of TNF-α in a positive feedback manner to cause further injury in the mucosa of colon. MMP-1 and TNF-α can be used clinically as biological markers to judge the severity of UC.

COMMENTS

Background

Matrix metalloproteinases (MMPs), their tissue inhibitors (TIMPs) and inflammatory cytokines, e.g., tumor necrosis factor-α (TNF-α) participate in the development of ulcerative colitis (UC) which is a chronic, non-specific inflammatory disease of the colon mucosa with unknown etiology and pathogenesis. This study was to deal with the expression of MMP-1 and TNF-α transcript and their proteins in colonic mucosa of patients with UC and their interrelationships in the pathogenesis of UC.

Research frontiers

Participation and functions of MMPs, TIMPs and inflammatory cytokines in the

pathogenesis of UC have been extensively studied in recent years. Study in this field has become one of the hotspots at present. Previous studies have demonstrated that MMPs and some inflammatory cytokines, such as TNF-α, are responsible for the development of ulceration and inflammation in the colonic mucosa of UC patients. Based on these findings, treatment targeting these proteins, such as anti-TNF-α antibody and exogenous MMPs inhibitors has been designed and studied in animal models. Preliminary results of these studies have shown beneficial and promising effects. Further experimental and clinical studies are needed before certain conclusions can be reached.

Innovations and breakthroughs

The association between MMPs and inflammatory cytokines with UC has been studied previously. However, most of the studies focused on their functions on the development of UC. The relationship between MMPs and other cytokines and the activity of UC remains largely unexplored. This study has bridged this gap and may provide additional targets for therapeutic development.

Applications

Since some basic evidence provided for MMP-1, TNF-α and their relationships in the development of UC, therapeutic approaches targeting MMPs or TNF-α, can be implemented in future study and new methods for treating UC may be developed.

Terminology

Matrix metalloproteinases (MMPs): MMPs are a group of zinc-dependent peptidases that degrade extracellular matrix (ECM). In this family, more than 20 MMPs have been identified. MMP-1, also known as interstitial collagenase, degrades mainly collagen type Ⅰ, Ⅱ, Ⅲ, Ⅵ, Ⅸ, and proteoglycan, and plays an important role in degrading ECM and in leading to colonic mucosa damages in UC patients.

Peer review

This is an informative study demonstrating the association between metalloproteinase (MMP) and tumor necrosis factor (TNF) with disease activity in individuals with ulcerative colitis. The association of TNF with UC is well known but the relationship of other cytokines with disease activity remains largely unexplored. This study is an attempt to bridge this gap and may provide additional targets for therapeutic development. The preliminary conclusion is justified and substantiated by the results obtained.

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RAPID COMMUNICATION

Small caliber overtube-assisted colonoscopy

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Abstract

AIM: To combine the benefits of a new thin flexible scope with elimination of excessive looping through the use of an overtube.

METHODS: Three separate retrospective series. Series 1: 25 consecutive male patients undergoing unsedated colonoscopy using the new device at a Veteran's hospital in the United States. Series 2: 75 male patients undergoing routine colonoscopy using an adult colonoscope, pediatric colonoscope, or the new device. Series 3: 35 patients who had incomplete colonoscopies using standard instruments.

RESULTS: Complete colonoscopy was achieved in all 25 patients in the unsedated series with a median cecal intubation time of 6 min and a median maximal pain score of 3 on a 0-10 scale. In the 75 routine cases, there was significantly less pain with the thin scope compared to standard adult and pediatric colonoscopes. Of the 35 patients in the previously incomplete colonoscopy series, 33 were completed with the new system.

CONCLUSION: Small caliber overtube-assisted colonoscopy is less painful than colonoscopy with standard adult and pediatric colonoscopes. Male patients could undergo unsedated colonoscopy with the new system with relatively little pain. The new device is also useful for most patients in whom colonoscopy cannot be completed with standard instruments.

Key words: Colonoscopy; Endoscopy; Colon Cancer; Colon cancer screening

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INTRODUCTION

Colonoscopy is typically performed using relatively large-diameter (11-13 mm) pediatric and adult instruments with enough rigidity to permit advancement of the instrument despite multiple turns within the bowel[1-3]. With these instruments, looping of the endoscope is a common difficulty that results in pain for the patient and hinders advancement of the endoscope[4,5]. In an effort to overcome looping, which is particularly common in the sigmoid colon, some practitioners have used stiffening overtubes that are preloaded on the back end of the scope and advanced over the colonoscope after negotiation of the sigmoid colon[6-8]. With the tube in place, further advancement of the instrument can be attained with minimal looping in the sigmoid; the overtube facilitates transmission of force from the endoscopist's pushing hand to the proximal end of the overtube. However, the overtubes employed for colonoscopy in the past have been relatively bulky and rigid devices that accommodate the large diameter of standard colonoscopes.

It is sometimes possible to perform colonoscopy using relatively thin and flexible upper endoscopes[9]. Thinner, more flexible scopes are often more easily advanced through the left colon[10]; this is perhaps the major reason why many endoscopists prefer pediatric colonoscopes over standard adult colonoscopes in female patients and in patients with sigmoid adhesions[2]. However, even pediatric colonoscopes are often associated with more difficulty in advancement through the proximal colon due to excessive looping[2]. These observations suggest that a very thin and flexible scope might facilitate insertion through the distal colon, but a mechanism to prevent excessive looping is important for optimal advancement through the proximal colon. One alternative to conventional colonoscopy that employs this strategy is to perform the procedure using a double balloon enteroscope[11-13]. The double balloon system also employs a very thin scope and an overtube, with the addition of balloons on the scope tip and overtube tip that can be inflated to secure the position by pressing against the bowel wall[14-16]. The double balloon system is used increasingly in patients who have failed conventional colonoscopy, but a major limitation is that the procedure is laborious and time consuming[17-19]. We surmised that by using a standard 160 cm length of scope, rather than the 200 cm long double balloon enteroscope, and a short 60 cm overtube, rather than a 140 cm long double balloon overtube, the procedure would be more efficient.

MATERIALS AND METHODS

The new colonoscopy system consists of a thin 9 mm scope, 170 cm in length, together with a 13 mm diameter 60 cm long overtube. The new 9 mm endoscope has the same outer diameter and instrument channel diameter (2.8 mm) as diagnostic upper endoscopes, but a 170 cm length that is similar to that of standard colonoscopes. The new scope has already received regulatory approval by the U.S. Food and Drug Administration for routine clinical use. The endoscope was provided by the Olympus corporation (Olympus America, Melville, New York, USA). The overtube (TS-13140, Fujinon Corporation, Wayne, New Jersey, USA) has a proprietary coating that reduces friction with the scope when the system is exposed to water; it is available commercially and is widely used in double balloon endoscopy. Because the overtube was too long, we cut off the proximal (near the hub) 100 cm and moved the plastic handle from its original position to the proximal end of the shortened tube (Figure 1). We also removed the inflatable latex balloon at the tip of the overtube because our earlier experience suggested that it is not generally helpful. Prior to each procedure, the overtube was temporarily filled with water to activate the lubrication system inherent in the tube and then back-loaded to the hub of the endoscope, leaving the distal 110 cm of the endoscope free for performing the initial portion of the examination without the overtube in place. After reaching the transverse colon, the scope was reduced, and the overtube was advanced over the scope until the handle on its proximal end was near the buttocks. An assistant then held the handle on the end of the overtube and the scope was advanced to the cecum.

This study consists of 3 retrospective series of patients undergoing colonoscopy at the Veterans Affairs Palo Alto Health Care System. Informed consent was obtained from all patients. The study was approved by the institutional review board of our hospital. All of the procedures were done by a single endoscopist with 8 years of experience performing approximately 1000 colonoscopies per year. The first series consisted of 25 consecutive male patients who were scheduled for unsedated colonoscopy (no medications given for the procedure); the patients were scheduled for unsedated procedures because of patient preference, medical contraindications to sedation, or lack of a driver to take them home after the procedure. The second series consisted of 75 consecutive male patients undergoing routine colonoscopy (3 female patients, 3 patients with previous partial colectomy and 1 patient with inflammatory bowel disease who necessitated a high-resolution magnification scope were not included in the series). An adult (Olympus CF-Q160AL), pediatric (Olympus PCF-Q180AL) and the thin scope/overtube were used in alternating cases. Patients were pre-medicated with lorazepam 2 mg sublingually (1 mg for patients over age 80) 15 min before the procedure. Patients were instructed by the nursing staff to request additional medication if they experienced pain or discomfort. Intravenous fentanyl was administered if the patient requested further sedation. The third series consisted of 35 patients who had incomplete colonoscopies in our endoscopy unit (the cecum was not reached) using any combination of standard adult (Olympus CF-Q160AL) and/or pediatric (Olympus PCF-160AL or PCF-Q180AL) endoscopes. The incomplete colonoscopies were performed by one of eight experienced attending endoscopists who work in our department.

Figure 1 The new 9 mm scope is shown alongside the 60 cm-long overtube.

Statistical analysis

Statistical comparison calculations were performed with two-tailed unequal-variance student's *t*-test[20]. Odds ratios and confidence intervals were calculated with the Newscombe-Wilson method without continuity correction[21].

RESULTS

In the first series, unsedated colonoscopy was successful in 25 consecutive patients at the Veterans Affairs Palo Alto Health Care System using the new device. None of the patients received any medication for the procedure. The indication for colonoscopy was a previous history of adenoma in 14 patients, positive stool occult blood in 3, screening in 2, family history of colon cancer in 2, hematochezia in 2, anemia in 1 and constipation in 1. Patients underwent unsedated colonoscopy for one of three reasons: patient preference (10 patients), inordinately high sedation risk (6) or unavailability of a driver to take them home after receiving sedation (9). All of the patients were male veterans. The age of the patients ranged between 53 and 94, with an average age of 68.1 and a median of 70.

Cecal intubation was achieved in all 25 patients, in a median time of 6 (average 6.4, range 2.5-15) min. Patients rated their maximal pain level during the procedure on a 0-10 scale. The median maximal pain level was 3 (average 2.9, range 0-6.5). Six patients had a maximal pain of 4 or higher. The entire procedure lasted a median time of 13 (average 13.6, range 7-28) min, including at least one snare polypectomy in 8 patients and forceps biopsy in another 2 patients. Small (< 10 mm) areas of mild erythema from passage of the overtube were seen occasionally on withdrawal, but no mucosal disruptions or other signs of trauma were observed. There was one complication:

bleeding one week after endoscopic mucosal resection of a 1.5 cm flat adenoma. The patient underwent urgent colonoscopy with successful clipping of an actively bleeding vessel at the resection site. He did not require blood transfusion or hospitalization.

The second series consisted of 75 male patients undergoing routine screening or surveillance colonoscopy. A standard adult colonoscope, pediatric colonoscope, and the thin scope/overtube system were used alternatingly; 25 procedures were performed with each type of scope. The median age of the thin scope group was 70 ± 10, compared to 69 ± 9 in the adult scope group (P = NS). The median age of the pediatric scope group was 65 ± 8, which was significantly younger than the thin scope group (P = 0.03).

Following premedication with lorazepam, 24/25 procedures with the thin scope were completed without additional sedation medication, compared to 9/25 with the adult scope (odds ratio 43, $P < 0.005$) and 14/25 with the pediatric scope (odds ratio 19, $P < 0.01$). The mean dose of fentanyl (μg) used was 12 ± 60 with the thin scope, compared to 51 ± 53 with the adult scope ($P < 0.05$) and 39 ± 53 (P = NS) with the pediatric scope. The median maximal pain during the procedure on a 0-10 scale was 3.5 ± 2 in the thin scope group, compared to 8 ± 2 in the adult colonoscope group ($P < 0.001$), and 7.5 ± 2.5 in the pediatric colonoscope group ($P < 0.001$). The cecum was reached in all patients, but the adult colonoscope was exchanged for a smaller diameter scope in 2 patients due to acute angulation in the sigmoid, and the pediatric colonoscope was exchanged for another scope in 2 patients due to excessive looping. The median time in minutes to reach the cecum was 5.5 ± 2.5 in the thin scope group, compared to 6.0 ± 2.1 in the adult colonoscope group (P = NS), and 4.0 ± 1.9 min in the pediatric colonoscope group (P = 0.004).

In the third series, 35 patients who had previously undergone unsuccessful colonoscopy (with inability to reach the cecum) had the procedure repeated using the new device. The reasons given by the endoscopist for the inability to reach the cecum were: excessive looping (22 patients), acute sigmoid angulation (11 patients) and acute angulation at the splenic flexure (2 patients). 28 of the patients were male and 7 were female. The age ranged between 33 and 90, with a median age of 65 and a standard deviation of 13. The procedure was successful in 33; the cecum could not be reached in 2 male patients due to excessive looping and double balloon colonoscopy was successfully performed in both of these cases. The median time to reach the cecum in the 33 successful cases was 7 (standard deviation 3.9) min. The median total colonoscopy time, including snare polypectomies in 8 patients and forceps biopsies in 3 patients, was 15 (standard deviation 8.4) min. There were no complications.

DISCUSSION

Sedation practices for colonoscopy vary widely across the world; unsedated colonoscopy is commonly performed in Asia and Finland[2], whereas it is generally very poorly accepted in the United States[22-25]. A major reason is pain due to looping of the endoscope. Small caliber overtube-assisted colonoscopy can potentially decrease looping and pain enough to make unsedated colonoscopy feasible in the general population. The small caliber scope used in this study was easily and rapidly advanced through the distal colon with minimal pain. After reduction of the scope, the thin low-friction overtube was advanced into position without significant resistance. With the overtube in place, it was generally possible to directly advance the endoscope to the cecum with relatively little attention to subsequent loop formation or paradoxical backward motion of the tip upon insertion. Our study suggests that this colonoscopy system could potentially make colonoscopy without intravenous sedation feasible a significant number of patients. The thin scope/overtube system was significantly less painful than conventional adult or pediatric colonoscopes. The 25 patients who required unsedated colonoscopy for a variety of indications all had successful procedures, and only 6 had a maximal pain level of 4 or higher on a 10 point scale. In the second patient series, when routine colonoscopy was performed after premedication with sublingual lorazepam, only 1 of 25 patients in the thin scope/overtube group requested additional sedation, compared to 11 of the patients with the pediatric colonoscope and 16 with the adult colonoscope. This suggests that most male patients undergoing routine screening or surveillance colonoscopy do not require intravenous conscious sedation and would be satisfied with a mild sedative that can be administered by mouth without an intravenous line. This could potentially result in a substantial cost savings by eliminating the need for extensive monitoring of patients receiving conscious sedation, and potentially make colonoscopy feasible for many patients in an office setting.

The thin scope/overtube system offers several benefits compared to standard colonoscopes. The thin scope is generally easily advanced through the sigmoid colon, as demonstrated by the successful performance of colonoscopy in 11 patients in the third series in whom previous colonoscopy was unsuccessful due to acute sigmoid angulations. Once the scope has been advanced through the left colon and reduction of loops has been performed, the overtube is advanced into position and subsequent looping of the scope during advancement through the right colon should theoretically be minimized. We did not specifically measure looping in the procedures we performed, but in our experience once the overtube was in place the scope was easily advanced through the right colon with little effort or attention required to prevent or reduce loops. The median time required to reach the cecum was 6 min in the unsedated group and 5.5 min in the lorazepam premedication group. This suggests that despite the additional step of positioning the overtube, reaching the cecum with the system can still be in an acceptable period of time. The median overall procedure time was 13 and 13.5 min in the unsedated and lorazepam groups, including at least one snare polypectomy in approximately 1/3 of the patients, demonstrating that withdrawal and polypectomy can also be performed efficiently.

Of the 35 patients who had previously failed colonoscopy using standard instruments, 33 had a

successful procedure with the thin scope/overtube system. The median time to reach the cecum in those 33 patients was only 7 ± 3.9 min. Although these cases were subjectively more difficult than routine cases, the patients received conscious sedation, which may facilitate rapid advancement, resulting in a similar overall time to cecum as in unsedated routine cases. This compares very favorably to our prior published experience of using a double balloon enteroscope to successfully complete 19 of 20 patients with previously incomplete colonoscopies, where the median time to reach the cecum was 28 ± 20 min[26]. Based largely on this difference in time, our preference is currently to use the thin scope/overtube system in all cases after failed colonoscopy with standard instruments, and reserve the double balloon enteroscope for those situations when the thin scope/overtube system is unsuccessful.

There are clear limitations to the current study: the retrospective design, the relatively small number of patients in each of the series, the overwhelmingly male patient population, the previously documented tolerance of male American veterans to unsedated colonoscopy[2,27], and the single-center design. Since the study was retrospective, the routine screening colonoscopy patients were not randomized to the new scope or a standard adult or pediatric scope, but rather the scopes were alternated. There were no complications attributable to the thin scope/overtube system in our study (the lone complication in the 3 retrospective series was a post-polypectomy bleed in one of the unsedated patients), but all of the procedures were performed by one experienced endoscopist and it remains to be demonstrated that the system is safe when used by practitioners of varying experience. Given the substantial differences across different institutions and different countries in the performance of unsedated colonoscopy, it is difficult to predict what effect this system could have on colonoscopy practice, but our study does demonstrate the potential for making colonoscopy less painful and better tolerated without dramatically increasing procedure time or complexity.

There are several disadvantages to the small caliber endoscope and overtube system used in this study. The overtube is marketed for single-use and is expensive in its current form (approximately US$200 at our institution); shortening the tube is also cumbersome. It is conceivable that a more reasonably priced short tube could be manufactured or that a reusable version could be developed. The 9-mm scope has a relatively small 2.8-mm channel which is adequate for typical maneuvers such as snare polypectomy and clip placement, but can limit suctioning of stool residue and resected polyps. A water jet port for efficient lavage is not available. The field of view, lighting and optical resolution may be slightly compromised compared to the latest generation of high-resolution adult colonoscopes. However, the potential for reducing pain may outweigh any of these disadvantages. Further studies will also need to address whether some colonoscopies are more difficult with this system, whether there is any increase in the rate of missed lesions, and whether certain therapeutic cases would be better served by using a standard colonoscope. The ultimate goal of reducing pain during colonoscopy enough to make unsedated colonoscopy better tolerated, thereby eliminating both complications due to sedation as well as an estimated 40% of the cost of the procedure[2], is particularly important given the current widespread screening practices in many countries. Additional adjunctive measures, such as using carbon dioxide instead of air for insufflation[28,29], may also play a role in achieving this goal.

COMMENTS

Background

Colonoscopy using standard instruments is often relatively painful and most procedures are done using intravenous sedation. Reduction of pain is a major focus of research because the potential for eliminating conscious sedation may make the procedure safer and less expensive.

Research frontiers

The development of new types of scopes for performance of colonoscopy with less pain and less sedation is a major area of research. Thinner scopes can potentially cause less pain during colonoscopy, but they can also result in more loop formation which can hamper the procedure.

Innovations and breakthroughs

In this article we describe our experience using a new thin scope in combination with an overtube designed to minimize loop formation. We demonstrate that the new system is less painful than standard colonoscopes.

Applications

This study suggests that the combination of a thin scope and an overtube can be useful for unsedated, routine and difficult colonoscopies.

Terminology

Looping: the process where the scope tip does not progress forward when the endoscopist pushes the scope into the patient, but rather the mid-section of the scope bows out, resulting in stretching of the colon.

Peer review

This is an important and well written contribution. Through retrospective comparative study, the authors concluded that small caliber overtube-assisted colonoscopy is less painful than colonoscopy with standard adult and pediatric colonoscopes. Male patients can undergo unsedated colonoscopy with the system with relatively little pain. The new device is also useful for most patients in whom colonoscopy cannot be completed with standard instruments.

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RAPID COMMUNICATION

Comprehensive screening for *reg1α* gene rules out association with tropical calcific pancreatitis

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Abstract

AIM: To investigate the allelic and haplotypic association of *reg1α* gene with tropical calcific pancreatitis (TCP). Since TCP is known to have a variable genetic basis, we investigated the interaction between mutations in the susceptibility genes, *SPINK1* and *CTSB* with *reg1α* polymorphisms.

METHODS: We analyzed the polymorphisms in the *reg1α* gene by sequencing the gene including its promoter region in 195 TCP patients and 150 ethnically matched controls, compared their allele and haplotype frequencies, and their association with the pathogenesis and pancreaticolithiasis in TCP and fibro-calculous pancreatic diabetes.

RESULTS: We found 8 reported and 2 novel polymorphisms including an insertion-deletion polymorphism in the promoter region of *reg1α*. None of the 5′ UTR variants altered any known transcription factor binding sites, neither did any show a statistically significant association with TCP. No association with any *reg1α* variants was observed on dichotomization of patients based on their N34S *SPINK1* or L26V *CTSB* status.

CONCLUSION: Polymorphisms in *reg1α* gene, including the regulatory variants singly or in combination with the known mutations in *SPINK1* and/or *CTSB* genes, are not associated with tropical calcific pancreatitis.

Key words: Tropical calcific pancreatitis; Lithostathine; Stone formation; Polymorphism; Haplotype

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INTRODUCTION

Chronic Pancreatitis (CP) is a continuing or relapsing inflammatory process of the pancreas resulting in exocrine and/or endocrine insufficiency. The cardinal manifestations of CP are pain, steatorrhoea, formation of pancreatic stones, and diabetes mellitus. Recently, mutations in cationic trypsinogen (*PRSS1*)[1], the serine protease inhibitor, Kazal type 1 (*SPINK1*)[2], and cystic fibrosis transmembrane regulator (*CFTR*)[3] genes have been found to be associated with chronic pancreatitis. Tropical calcific pancreatitis (TCP) is an idiopathic, juvenile, nonalcoholic form of chronic pancreatitis with a unique tropical distribution, while fibro-calculous pancreatic diabetes (FCPD) is a condition characterized by the development of diabetes secondary to TCP. A genetic etiology for TCP and FCPD was suggested by Pitchumoni *et al*[4] and confirmed by Mohan *el al*[5], who showed familial aggregation of FCPD with evidence of vertical transmission in some families. We previously reported evidence of its genetic nature, based on clustering of TCP in a few families and its association with SPINK1 mutations[6].

In a previous study we had shown that mutations in *PRSS1* did not play a role in TCP, whereas mutations in *SPINK1* gene were found in the majority of such patients[7]. Recently, we have demonstrated that mutations in pro-peptide region of cathepsin B (*CTSB*) gene are strongly associated with TCP[8]. Irrespective of mutations in different genes, premature intra-pancreatic activation of trypsinogen is believed to play a central role in the pathogenesis of chronic pancreatitis. However, the phenomenon of stone formation continues to be poorly understood. Although various hypotheses have been proposed for stone formation, the development of protein plugs appears to be an important initiating event[9]. It has been proposed that if concentration-dependent precipitation is the cause of protein plug formation, there should be an associated increase in the concentration of some proteins in the pancreatic juice[10].

Lithostathine C was initially isolated as a major

proteic component of pancreatic stones in alcoholic calcifying chronic pancreatitis, and was consequently called pancreatic stone proteic (PSP)[9]. Human PSP or Reg protein is encoded by *reg1α* gene (regenerating gene)[11] as a 166 amino acid pre-proprotein with a 22-residue long signal sequence. A similar protein with 89% homology with PSP is coded by another gene *reg1β* belonging to the same type 1 subclass but has never been isolated and its expression in pancreas remains controversial[12]. Only the Reg1α protein is highly represented in the human pancreatic secretions[13] and is found to be 100% identical to a glycoprotein that is generated by trypsin cleavage resulting in a 133 aa polypeptide previously named pancreatic thread protein (PTP). The mature protein is a soluble glycoprotein existing under 11 isoforms (17-22 kDa)[14], generated by post-translational modification such as glycosylation. Of these isoforms, S2-S5 are believed to inhibit calcite crystal growth *in vitro* and thus stone formation[15,16]. PSP is highly susceptible to trypsin cleavage at Arg11-Ile12 bond resulting in PTP formation, which is known to form fibrilla at neutral pH and is found in protein plugs or stones extracted from pancreatic ducts of CP patients[9,17]. The exact function of Reg1α protein is not clear, but it could stimulate the regeneration and/or growth of pancreatic β-cell[18]. We hypothesized that mutations in the promoter region of *reg1α* may lead to altered expression of the protein. Alternatively, variants in the coding region could predispose the Reg1 protein to increased tryptic cleavage resulting in greater formation of PTP. This may cause precipitation of PTP and obstruction of the pancreatic duct secondary to protein plugs and calculi, resulting in pancreatitis. Since high levels of intrapancreatic trypsin produced both by known mechanisms like *PRSS1* mutations or by as yet unknown mechanisms such as mutations in *SPINK1* and *CTSB* genes is an established fact, it can be speculated that intrapancreatic trypsin may cleave the soluble lithostathine (PSP S2-S5) into insoluble PTP. FCPD is a condition characterized by the development of diabetes secondary to TCP, however, the etiology of diabetes in these patients is not clear, hence we investigated the role of these polymorphisms in the pathogenesis of FCPD. Since, N34S *SPINK1* mutations occur in the majority of these patients and it is not clear whether pancreatitis is the cause or the effect of ductal obstruction, we attempted to investigate the interaction between N34S *SPINK1* mutation and L26V *CTSB* mutations and *reg1α* gene polymorphisms. We also performed haplotype analysis to see if a particular *reg1α* haplotype is associated with the disease.

MATERIALS AND METHODS

Patients and controls

195 unrelated subjects belonging to Australoid ethnicity[19] (134 males and 61 females), diagnosed with tropical calcific pancreatitis at the Asian Institute of Gastroenterology, Hyderabad and 150 age and sex matched individuals (98 males and 52 females) of the same ethnicity but without any evidence of pancreatitis on imaging studies were included as patients and controls respectively[7]. Both the patients and the controls completed a detailed questionnaire and underwent similar investigations including imaging studies. Written informed consent was obtained from all the patients and controls, before the collection of blood samples. The Institutional Ethics Committee of both participating institutes approved the study as per the guidelines of the Indian Council of Medical Research for research on human subjects.

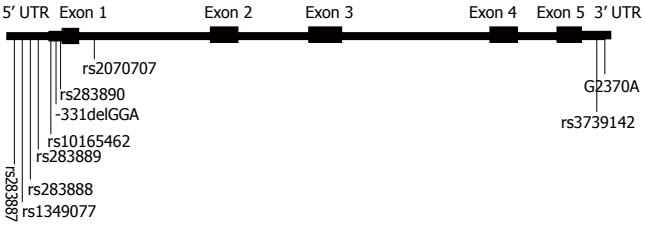

Figure 1 Diagrammatic representation of the *reg1α* gene showing exons (translated), UTRs (untranslated regions) and the location of the polymorphisms studied (constructed on the lines of *reg1α* gene structure as on UCSC genome browser, figure not to scale).

Table 1 Primer sequences and PCR conditions for the *reg1α* gene

Primer	Sequence (5'-3')	T_{ann} (℃)
1F	TGTCCCAATTCATATACTTA	50
1R	GCATGTTAGAGACGCCCTTC	
2F	CGGGAAAAGGCTCGTACTGG	60
2R	TCAGTTCTCCACCCCATTAG	
3F	TAAAAGGGAAACTGGAGACT	56
3R	CCTCCTTCTTACTTCTCAAA	
4F	TGCACTGTAGATGATTGGAG	62
4R	AAAGACTGGGGTAGGTAAAACT	
4F-INT1	TCTTGGTGGAATACAGTTAA	Seq
4F-INT2	AATGGATGTTTGGTTTTTGT	Seq

F: Forward; R: Reverse; T_{ann}: Annealing temperature; INT: Internal primer for sequencing.

Genetic analysis

Genomic DNA was isolated from patients and healthy volunteers using salting out method[20]. The human *reg1α* gene is located on 2p12 with six exons (5 translated exons, Figure 1) spanning 2962 base pairs and is known to contain TATA and CCAAT box-like sequences that are located at 27 and 100 bp upstream from the transcriptional initiation site[21]. Using the software tool Transplorer (Biobase Biological Databases, Wolfenbuttel, Germany), we attempted to identify transcription factor binding sites in a sequence of about 1600 bases upstream of transcriptional start site, which included the above-mentioned sequence[22]. We screened the complete *reg1α* gene including its exons, introns and 5'- and 3'- untranslated regions by direct sequencing, using 4 sets of primers in 50 patients and 50 controls (Table 1). PCR products were purified and sequenced individually on both the strands using Big-dye terminator cycle sequencing ready kit (Applied Biosystems, Foster City, CA) on an ABI3730 Genetic Analyzer (Applied Biosystems). In case of unclear sequence data, we repeated sequencing under various conditions until the genotype was determined correctly. Six SNPs (Table 2) that

Table 2 Distribution of polymorphisms in *reg1α* gene in patients with tropical calcific pancreatitis and healthy controls

Polymorphism[2]	rs number	Position[5]	Minor allele frequency		OR (95% CI)	*P* Value
			Patients (*n* = 195)	Controls (*n* = 150)		
G-974C[4]	rs283887	79200522	0.01	0.02	0.49 (0.02-7.10)	1.00[1]
G-938A	rs1349077	79200558	0.34	0.33	1.05 (0.56-1.96)	0.88
T-912G	rs283888	79200584	0.49	0.50	0.94 (0.54-1.78)	0.84
G-501A[4]	rs283889	79200995	0.01	0.02	0.49 (0.02-7.10)	1.00[1]
T-385C	rs10165462	79201111	0.32	0.29	1.15 (0.60-2.20)	0.65
-331delGGA[3]	-	-	0.01	0.01	1.00	1.00[1]
T-243G	rs283890	79201253	0.34	0.35	1.09 (0.75-1.58)	0.63
G209T	rs2070707	79201704	0.20	0.17	1.29 (0.78-2.12)	0.29
G2199A	rs3739142	79203694	0.34	0.34	1.01 (0.70-1.48)	0.94
G2370A[3,4]	-	-	0.01	0.03	0.33 (0.01-3.60)	0.61[1]

AA: Amino acid; OR: Odds ratio; CI: Confidence interval; [1]Yates corrected P value; [2]Nomenclature as per NCBI sequence Accession No. NT_022184; [3]Novel polymorphism; [4]Data from 50 patients & 50 controls; [5]Chromosomal location according to UCSC Genome Browser, March 2006 build (dbSNP build 126).

Table 3 Comparison of *reg1α* gene polymorphisms in FCPD and TCP patients, and controls

SNP[1]	Minor allele frequency			FCPD *vs* TCP		FCPD *vs* Controls		TCP *vs* Controls	
	FCPD (*n* = 94)	TCP (*n* = 101)	Controls (*n* = 150)	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
G-938A	0.36	0.32	0.33	1.20 (0.64-2.24)	0.55	1.14 (0.61-2.13)	0.66	0.96 (0.51-1.80)	0.88
T-912G	0.47	0.51	0.50	0.85 (0.47-1.54)	0.57	0.89 (0.49-1.60)	0.67	1.04 (0.58-1.88)	0.89
T-385C	0.31	0.30	0.29	1.05 (0.55-2.00)	0.88	1.10 (0.57-2.11)	0.76	1.05 (0.55-2.02)	0.88
T-243G	0.33	0.35	0.35	0.91 (0.49-1.71)	0.77	0.91 (0.49-1.71)	0.77	1.00	1.00
G209T	0.24	0.17	0.17	1.54 (0.73-3.27)	0.22	1.54 (0.73-3.27)	0.22	1.00	1.00
G2199A	0.41	0.33	0.34	1.41 (0.76-2.62)	0.24	1.35 (0.73-2.50)	0.31	0.96 (0.51-1.79)	0.88

SNP: Single nucleotide polymorphism; n: Number of individuals; OR: Odds ratio; CI: Confidence interval; TCP: Tropical calcific pancreatitis; FCPD: Fibrocalculous pancreatic diabetes; [1]Only SNPs with > 3% minor allele frequency have been presented; The minor allele frequency at each polymorphism was compared between the three groups and *P* value with OR and 95% CI were calculated.

exceeded allele frequency of 3% were screened in another 145 patients and 100 controls from the same ethnic background. N34S and L26V mutations in the *SPINK1* and in *CTSB* genes respectively were analyzed using the methodology as described previously[7,8]. Ten percent of randomly chosen samples were re-genotyped for validation of the data, and no genotyping error was noted.

Statistical analysis

The allele and genotype frequencies were calculated for each polymorphism (Table 2) in the whole cohort as well as in TCP and FCPD patients separately (Table 3). We analyzed any deviation from the Hardy-Weinberg equilibrium, and observed the expected genotype frequencies by Markov simulation based goodness of fit test using Arlequin software version 2[23]. Pearson's Chi-square and Yates corrected chi-square test were used to analyze the statistical significance of the difference in allelic distribution of polymorphisms in patients and controls. Haplotypes were generated with 6 polymorphisms having a minimum allele frequency greater than 3% with the accelerated Expectation-maximization algorithm using Haploview software (Version 3.2) and compared the results between patients and controls[24]. This study was 90% powered to detect a relative risk of 1.60 (http://www.dssresearch.com/). Unless indicated specifically, a *P*-value of 0.05 was considered significant in all the analyses. Chi-square, genotype relative risk, odds ratio and confidence interval were calculated using the PEPI (Programme for EPIdemiologists, ver 4.04) and DeFenetti programs (http://www.ihg.gsf.de/cgi-bin/hw/hwa1/).

RESULTS

We initially sequenced complete *reg1α* gene in 50 patients and an equal number of controls and subsequently, additional patients and controls were screened for six SNPs with rare allele frequency of > 3%. Sequencing results revealed the presence of 8 reported SNPs, one novel SNP and one insertion-deletion polymorphism in the promoter region of the gene (Table 4). We did not observe any significant deviation from Hardy-Weinberg equilibrium ($P > 0.05$) for any of the polymorphisms. The polymorphisms in the promoter region were of prime interest, since the levels of *reg1α* expression differ considerably between the pancreas of patients and controls. Transplorer predicted 3 transcription factor-binding sites (C-Rel, -1513 to -1609; NFκB2, -1527 to -1614; and Hesx1, -15 to -105) within the region +10 to -1600 bp of the putative promoter region[21]. We sequenced the upstream region flanking the 5'-UTR (about 1176 bp upstream of translation start site) along with putative promoter region and found four reported SNPs, G-938A, T-912G, T-385C, T-243G which were equally frequent in patients and controls. A novel insertion-deletion polymorphism at -331 position (-331 to -329) involving

Table 4 Genotype data of polymorphisms analyzed in *reg1α* gene

Polymorphism	Patients (n = 195)			Controls (n = 150)		
	AA	Aa	aa	AA	Aa	aa
G-974C[1]	49	1	0	48	2	0
G-938A	92	73	30	74	52	24
T-912G	53	93	49	42	65	43
G-501A[1]	49	1	0	48	2	0
T-385C	98	76	21	80	52	18
-331delGGA	193	2	0	148	2	0
T-243G	92	73	30	69	58	23
G209T	125	61	9	103	43	4
G2199A	94	68	33	72	54	24
G2370A[1]	49	1	0	47	3	0

AA: Homozygous for major allele; Aa: Heterozygous; aa: Homozygous for minor allele. [1]Data from 50 patients & 50 controls.

Table 5 Haplotype frequencies of *reg1α* gene in patients with tropical calcific pancreatitis and healthy controls

S. No.	Haplotype	Haplotype frequency (%)		OR	P
		Patients (n = 195)	Controls (n = 150)	(95% CI)	value
1	GGTGGG	43.1	43.3	~1	~1
2	ATCTGA	30.3	31.3	0.95 (0.5-1.82)	0.88
3	GTTGTG	19.0	17.3	1.15 (0.52-2.5)	0.33
4	GGTTGG	2.1	2.0	~1	~1

OR: Odds ratio; 95% CI: 95% confidence interval; Haplotypes generated using six SNPs with minor allele frequency of > 3%, haplotypes with frequency > 2% are presented; Order of SNPs; G-938A, T-912G, T-385C, T-243G, G209T, G2199A in the reference sequence.

Table 6 Distribution of *reg1α* gene polymorphisms in tropical calcific pancreatitis patients based on N34S *SPINK1* and L26V *CTSB* status

SNP	*SPINK1* mutation				*CTSB* mutation			
	Minor allele frequency[1]		OR (95% CI)	P value	Minor allele frequency[2]		OR (95% CI)	P value
	N34S (n = 48)	WILD (n = 82)			L26V (n = 105)	WILD (n = 73)		
G-938A	0.33	0.34	0.96 (0.51-1.79)	0.88	0.34	0.31	1.15 (0.61-2.16)	0.65
T-912G	0.49	0.45	1.17 (0.65-2.13)	0.57	0.43	0.54	0.64 (0.35-1.17))	0.12
T-385C	0.31	0.33	0.91 (0.48-1.73)	0.76	0.31	0.26	1.28 (0.66-2.48)	0.43
T-243G	0.33	0.39	0.77 (0.41-1.43)	0.38	0.34	0.31	1.15 (0.61-2.16)	0.65
G209T	0.18	0.21	0.83 (0.39-1.76)	0.59	0.23	0.19	1.27 (0.61-2.66)	0.49
G2199A	0.34	0.35	0.96 (0.51-1.79)	0.88	0.38	0.27	1.66 (0.87-3.15)	0.10

SNP: Single nucleotide polymorphism; n: Number of individuals; OR: Odds ratio; CI: Confidence interval; [1]Minor allele frequency based on N34S *SPINK1* status; [2]Allele frequency based on L26V *CTSB* status.

deletion of GGA (-331delGGA) in the 5'UTR was identified but the frequency of deletion allele was similar in cases and controls. None of the seven polymorphisms in the promoter region altered the transcription-binding site and hence neither any existing transcription binding site was destroyed nor was a new site created. Other SNPs included two in the intronic region and one in the 3' UTR region of *reg1α* gene. All ten polymorphisms had comparable allele frequencies in patients and controls and the difference was statistically not significant (Table 2). Allelic odds ratio and confidence interval did not indicate an association with any of the polymorphisms identified in *reg1α* with TCP (Table 2). Haplotype analysis using the six *reg1α* polymorphisms with greater than 3% minor allele frequency supported the observations made from the allelic and genotypic data at different polymorphisms (Table 5). The patient population was divided into FCPD and TCP patients based on the presence or absence of diabetes, but we failed to observe any association between FCPD and polymorphisms in *reg1α* gene (Table 3). We also dichotomized the patient population based on the presence or absence of N34S mutation in the *SPINK1* gene and L26V mutation in the cathepsin B gene and compared the allele frequency of 6 SNPs in *reg1α* gene of patients having at least one mutant allele with those with the wild type pattern at the above mentioned mutations (Table 6), but could not detect any interaction between them and the *reg1α* variants.

DISCUSSION

TCP is associated with the presence of large calculi throughout the main pancreatic duct[25,26]. However, the mechanism of stone formation is not completely understood[26]. A decrease in tissular pancreatic stone protein mRNA concentration is associated with CCP[27,28]. The role of Reg proteins is debatable but they are known to be associated with pancreatic islet regeneration, diabetogenesis and amelioration of surgical diabetes in animal models[18]. Its role in pancreatic stone formation is not clear with suggestions that lithostathine could promote the nucleation of calcite crystals or may prevent pancreatic lithiasis by inhibiting calcite crystal nucleation and growth in the pancreatic juice[29]. Thus, mutations in *reg1α* gene could play an important role in the pathogenesis of TCP and FCPD.

A previous study, analyzed the exons of *reg1α* gene using a combination of Restriction fragment length polymorphism (RFLP), Single strand conformation polymorphism (SSCP) and sequencing techniques in 50 FCPD patients and controls, but did not identify any nucleotide substitutions and ruled out any contribution

of mutations in the coding regions of *reg1α* gene[30]. However, these workers did speculate about a possible role of regulatory variants in *reg1α* gene. A subsequent study also analyzed only the coding region in 12 Thai FCPD patients and 22 controls and ruled out any association with the disease[31]. T-385C, a polymorphism in exon 1 (5'UTR) with a moderately high allele frequency (0.32 in patients and 0.29 in controls) could have been missed in these studies due to the inherent limitations of techniques like SSCP in detecting any sequence changes. Our study involving extensive analysis of the gene as well as of the promoter region detected several polymorphisms including the promoter variants but the results suggest that there may not be any allelic or haplotypic association between the polymorphisms in *reg1α* and TCP.

As the *reg1α* gene is believed to be involved in islet cell repair and regeneration[18], we examined the association of *reg1α* variants with TCP and FCPD. The etiology and relationship of diabetes mellitus in FCPD are not well understood. Some believe that diabetes in FCPD is secondary to TCP while others suggest there is selective β-cell impairment, the latter hypothesis is supported by the occurrence of FCPD in some patients at a very young age. Evidence showing a preserved pancreatic α-cell function in diabetics with advanced chronic pancreatitis of the tropics indicates the presence of two different pathogenic mechanisms, one causing chronic pancreatitis and the other selective pancreatic β-cell impairment and subsequently diabetes mellitus[32]. However, an independent analysis of the TCP and FCPD patients did not suggest any role for *reg1α* variants in FCPD patients. Although, nearly one-half of the TCP patients carry N34S *SPINK1* mutation and the mutations in *SPINK1* and *CTSB* are the only genetic changes known to be associated with TCP, we did not find any evidence of an interaction between them. Although the present study had limited power to analyse such an interaction, our preliminary observations did not find a statistically significant difference in allele frequency between these groups for any polymorphism, suggesting the lack of epistatic interaction between *SPINK1* and/or *CTSB* with *reg1α* gene.

In conclusion, polymorphisms in *reg1α* gene, including those in the regulatory region are unlikely to contribute to the pathogenesis of pancreaticolithogenesis in tropical calcific pancreatitis. Other genes such as those involved in calcium signaling and regulation, either interacting with *reg* genes or functioning independently may play a role in stone formation in tropical calcific pancreatitis.

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COMMENTS

Background

Chronic pancreatitis (CP), an inflammatory condition of the pancreas with diverse etiologies, is usually associated with parenchymal calcification and presence of stones in the pancreatic duct. The process of stone formation in chronic pancreatitis is not completely understood. Lithostathine (encoded by *reg1α* gene), identified as a major proteic component of pancreatic stones in patients with alcoholic calcifying chronic pancreatitis, is thought to play an important role in the inhibition of stone formation and its levels are known to correlate with disease severity and is possibly regulated by the *reg1α* variants.

Research frontiers

Tropical calcific pancreatitis (TCP) and fibrocalculous pancreatic diabetes (FCPD; TCP presenting with diabetes) is a type of chronic pancreatitis specific to tropical countries. One of the important features of this condition is formation of large and irregular intraductal stones. Currently, there is considerable interest in understanding the mechanism of stone formation, the factors that inhibit stones, the genes involved in the process of pancreaticolithiasis as well as the effect of various polymorphisms. An additional area of interest is the relationship between the pancreatic inflammation and pancreaticolithiasis as well as the influence of genetic variants that predict susceptibility to the development of chronic pancreatitis.

Innovations and breakthroughs

The present study attempted to open new frontiers in the area of molecular pathogenesis of stone formation in TCP and FCPD by ruling out the role of *reg1α* variants in pancreaticolithiasis.

Applications

The results of the present study propose a new assessment of the pathogenesis of stone formation in TCP and FCPD. Further studies should be designed to elucidate more information.

Terminology

The process of stone formation, lithogenesis, is believed to be initiated by calcite nucleation with the subsequent deposition of proteins leading to protein plug formation; Lithostathine C is known to influence this process.

Peer review

The authors of this manuscript screened the *reg1α* gene including the regulatory region by sequencing and examining the association of the polymorphisms in the gene with pancreaticolithiasis in TCP and FCPD. The authors conclude that neither the previously reported nor novel variants in the *reg1α* gene predict the susceptibility to pancreaticolithiasis in TCP and FCPD.

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RAPID COMMUNICATION

In-vitro activation of cytotoxic T lymphocytes by fusion of mouse hepatocellular carcinoma cells and lymphotactin gene-modified dendritic cells

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Abstract

AIM: To investigate the *in-vitro* activation of cytotoxic T lymphocytes (CTLs) by fusion of mouse hepatocellular carcinoma (HCC) cells and lymphotactin gene-modified dendritic cells (DCs).

METHODS: Lymphotactin gene modified DCs (DCLptn) were prepared by lymphotactin recombinant adenovirus transduction of mature DCs which differentiated from mouse bone marrow cells by stimulation with granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-4 (IL-4) and tumor necrosis factor alpha (TNF-α). DCLptn and H22 fusion was prepared using 50% PEG. Lymphotactin gene and protein expression levels were measured by RT-PCR and ELISA, respectively. Lymphotactin chemotactic responses were examined by *in-vitro* chemotaxis assay. *In-vitro* activation of CTLs by DCLptn/H22 fusion was measured by detecting CD25 expression and cytokine production after autologous T cell stimulation. Cytotoxic function of activated T lymphocytes stimulated with DCLptn/H22 cells was determined by LDH cytotoxicity assay.

RESULTS: Lymphotactin gene could be efficiently transduced to DCs by adenovirus vector and showed an effective biological activity. After fusion, the hybrid DCLptn/H22 cells acquired the phenotypes of both DCLptn and H22 cells. In T cell proliferation assay, flow cytometry showed a very high CD25 expression, and cytokine release assay showed a significantly higher concentration of IFN-γ and IL-2 in DCLptn/H22 group than in DCLptn, DCLptn+H22, DC/H22 or H22 groups. Cytotoxicity assay revealed that T cells derived from DCLptn/H22 group had much higher anti-tumor activity than those derived from DCLptn, H22, DCLptn+H22, DC/H22 groups.

CONCLUSION: Lymphotactin gene-modified dendritoma induces T-cell proliferation and strong CTL reaction against allogenic HCC cells. Immunization-engineered fusion hybrid vaccine is an attractive strategy in prevention and treatment of HCC metastases.

Key words: Hepatocellular carcinoma; Dendritic cell; Cytotoxic T lymphocyte

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INTRODUCTION

Dendritic cells (DCs) are the most important antigen-presenting cells (APCs)[1-3]. DCs-based vaccinations have been demonstrated to be effective in inducing antigen-specific cytotoxic T lymphocyte (CTL) responses[4-9]. Previous studies in mouse tumor models or cancer patients demonstrated that vaccination with hybridomas from tumor cells and DCs induces regression of established carcinomas, lymphomas and myeloma[10-16]. This study was to investigate the *in-vitro* immune effects of fusion of mouse hepatocellular carcinoma (HCC) cells and lymphotactin (Lptn) gene-modified DCs and its antitumor activity.

MATERIALS AND METHODS

Animals, recombinant adenoviruses and cell lines

Five- to six-week old Female BALB/c (H-2K^d) mice were obtained from the Animal Resource Center of Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, and maintained in specific pathogen-free conditions for use at the age of 6-8 wk. Recombinant Ad5 adenoviruses harbouring mouse lymphotactin (AdLptn) or LaZ gene (AdLacZ) were kindly provided by Dr. Cao Xue-Tao. The recombinant adenoviruses were propagated in

human embryonic kidney 293 (HEK293) cells, and purified by cesium chloride (CsCL) density gradient centrifugation. Titers of AdLptn and AdLacZ determined by plaque assay on HEK293 cells were 3.6×10^9 plaque-forming units (PFU)/mL and 4.5×10^9 PFU/mL, respectively. H22 cells, established as a BALB/c mouse origin HCC cell line, were purchased from China Center for Type Culture Collection. All the cells were cultured in RPMI-1640 (H22 cells) or DMEM (HEK293 cells) medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

DC culture

DCs were prepared as previously described[17] with certain modifications. Briefly, bone marrow cells prepared from femora and tibias of normal BALB/c mice were depleted of red blood cells with ammonium chloride and plated in RPMI-1640 plus 10% FCS and 10 ng/mL granulocyte/macrophage colony-stimulating factor (GM-CSF; R&D) with conjunction of 10 ng/mL interleukin-4 (IL-4; R&D) on d 1. On d 3, nonadherent granulocytes, T and B cells were gently removed and fresh media were added. On d 5, loosely adherent proliferating DC aggregates were dislodged and re-plated in the fresh media, and supplemented with 50 ng/mL tumor necrosis factor-α (TNF-α; R&D). On d 7, the released nonadherent mature DCs were harvested. CD11c-positive DCs accounted for more than 80% of the harvested cells as measured by flow cytometry.

Adenovirus transduction

Cultured DCs were pelleted and washed with PBS prior to the addition of virus. Virus stock (stored at -80℃) was thawed at room temperature and diluted in serum-free RPMI-1640 medium. The pellets of DCs were resuspended in serum-free RPMI-1640 and virus was added. After 2 h incubation with virus, cells were washed once in PBS. DCs were resuspended in a cytokine-supplemented medium which was retained after DC culture. Twenty-four hours after gene modification, LacZ gene-modified DCs (DCLacZ) were collected for X-gal staining to evaluate the gene transfer efficiency. Lymphotactin gene-modified DCs (DCLptn) were collected for phenotypic analysis and fused with H22 cells *in vitro*.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from cells using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. cDNA was prepared from total RNA using a hexanucleotide random primer and SuperScrip Moloney murine leukemia virus reverse transcriptase (Life Technologies). PCR primers for the amplification of mouse lymphotactin and beta-actin used are as follows (lymphotactin forward primer: 5'TGGG GACTGAAGTCCTAGAAG3'; reverse primer: 5'TTA CCCAGTCAGGGTTACTGCTGCTGTG3', with the product size of 300 bp. Beta-actin forward primer: 5'TG GAATCCTGTGGCATCCATGAAAC3'; reverse primer: 5'TAAAAGCCAGCTCAGTAACAGTCCG3', with the expected size of 359 bp.). PCR was performed in a Perkin Elmer Cetus DNA thermal cycler using Taq DNA polymerase (Life Technologies). The program consisted of 25 cycles of template denaturation at 94℃ for 1 min, annealing of primers at 60℃ for 1 min and synthesis at 72℃ for 2 min, followed by a final extension at 72℃ for 10 min. The PCR products were analyzed by agarose gel electrophoresis. Controls without reverse transcriptase were used to confirm that the RT-PCR products obtained were not the result of contamination with genomic DNA.

ELISA for measuring lymphotactin in supernatants

Lymphotactin protein in the supernatants from DCLptn was quantitatively determined with a commercial "sandwich" enzyme immunoassay kit (R&D) according to the manufacture's instructions. Briefly, Costar EIA microplates were coated with 100 μL of 2 μg/mL rat-anti-mouse lymphotactin as a capture antibody, incubated overnight at room temperature, and blocked with 1% bovine serum albumin (BSA) in PBS. Then, 100 μL of serially diluted standards or culture supernatant samples was added in triplicate and incubated at room temperature for 2 h. The plates were washed and incubated at room temperature for 2 h with 100 μL of 400 ng/mL biotinylated goat anti-mouse lymphotactin as a detection antibody. After washing, wells were incubated for 20 min in 100 μL of streptavidin- horseradish peroxidase (HRP) solution, and developed with substrate solution.

Cell fusion

DCLptn were fused with tumor cells at a 3:1 (DC: tumor) ratio using 50% polyethylene glycol (PEG, 50% PEG/10% DMSO in PBS, Sigma). In brief, H22 cells were inactivated by 30 μg/mL mitomycin, washed and mixed with DCLptn. After centrifugation, 1 mL of 50% PEG was added to the cell pellets for 2 min at 37℃. Then, an additional 10 mL of warm serum-free medium was added to dilute PEG over the next 3 min with continuous stirring. PEG-treated cells were centrifuged at 400 × *g* for 5 min, resuspended with RPMI-1640 medium supplemented with 20% FCS, 10 ng/mL GM-CSF and 10 ng/mL IL-4, and cultured overnight.

To determine the efficiency of cell fusion, H22 cells were stained with PKH-26 (red fluorescence, Sigma) and DCLptn were stained with PKH-2 (green fluorescence, Sigma). The cells stained with the fluorescence dyes were treated with PEG and cultured overnight as described above. On the next day, the stained cells were analyzed using a FACScan flow cytometer (Becton Dickinson) under a confocal microscope.

Phenotypic analysis

After washing, cells were resuspended in PBS containing 1% BSA, and stained with fluorescence-conjugated monoclonal antibody (H-$2K^d$, I-A^d, CD80, CD86, CD40, CD54) or isotype control antibody for 30 min at 4℃. The stained cells were washed and analyzed using FACS

In vitro chemotaxis assay

Chemotactic responses of lymphotactin to T cells were examined using modified boyden microchemotaxis chambers (Neuro Probe, Gaithersburg) and polyvinyl pyrrolidone-free 5 μm pore size polycarbonate

membranesy. Briefly, spleen cells from naïve BALB/C mice were used as effector cells. The bottom wells of the chamber were loaded with supernants of H22, DC, DCLptn, DCLacZ or RPMI-1640 alone, and the upper wells contained 1×10^5 effector cells. After 1 h incubation and staining, data were obtained by counting five nonoverlapping high power microscopic fields from each well. Cells were considered chemoattracted if the chemotactic index (number of cells migrating in experimental well/number of cells migrating in RPMI-1640 medium only) was greater than 2.

CD25 expression and cytokine production after autologous T cell stimulation

To determine the proliferation and differentiation of lymphocytes, CD25 expression and cytokine production after autologous T cell stimulation were assayed. Briefly, spleen cells from naïve BALB/C mice were passed over nylon wool with their purity determined by FACS (percentage of $CD3^+$ cells near 90%) and used as responder cells at 1×10^5/well in 96-well U-bottom plates. Syngeneic H22, DCLptn, H22+ DCLptn (H22 cells co-cultured with DCLptn at a ratio of 3:1), DC/H22 (H22 cells fused with DC at a ratio of 3:1) and DCLptn/H22 (H22 cells fused with DCLptn at a ratio of 3:1) cells were inactivated with 30 μg/mL mitomycin for 30 min and added to responder cells in varying cell numbers. Cells were cultured at 37℃ in RPMI-1640 medium containing 10% FCS and 5% CO_2 for 2 d. Control wells contained T cells alone. At the end of experiment, supernatants were harvested for cytokine production assay by ELISA and co-cultured T-cells were collected for analyzing CD25 expression by FACS.

CTL assay

Cytotoxic function of the activated T lymphocytes stimulated with DCLptn/H22 was determined by cytotoxicity test. Inactivated cells were co-cultured with spleen T cells separated from naïve BALB/C mice at a 1:10 ratio in the presence of 20 U/mL mouse IL-2 for 7 d. The stimulated T cells were isolated and used as effector cells in lactate dehydrogenase (LDH, Roche) cytotoxicity assay. H22 cells were used as target cells. All steps were performed following the manufacturer's instructions. Briefly, after washed with assay medium (RPMI1640 with 1%BSA), the effector cells were co-cultured at 37℃ with target cells in a 96-well round bottom plate for 6 h, then the plate was centrifuged and the supernatants were transferred to another flat-bottom ELISA plate. One hundred μL of LDH detection mixture was added to each well and incubated at room temperature in the dark for 30 min. Absorbance was measured with an ELISA reader at 490 nm. The spontaneous release of LDH by target cells or effector cells was assayed by incubation of target cells in the absence of effector cells and vice versa, the maximum release of LDH was determined by incubation of the target cells in 1% Triton X-100 in assay medium. The percentage of cell-mediated cytotoxicity was determined by the following equation: cytotoxicity (%) = [(mixture of effectors and targets-effector control)/(maximum-spontaneous)] × 100.

Statistical analysis

Data were expressed as mean ± SD. Experiment results were analyzed using SPSS 10.0 statistical package. Differences among groups were assessed by the Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Lymphotactin expression and functional assay

DC and H22 did not express any detectable Lptn, which was detected in DCLptn and H22Lptn (Figure 1). The results indicate that adenovirus vector could effectively transducer the Lptn gene.

In order to quantitatively determine Lptn protein in supernatants from gene-modified DCs, culture supernatants were harvested and determined for Lptn production by ELISA. The results showed that about 0.35 ± 0.04 ng/mL Lptn could be detected in the supernatants of DCLptn, while nearly no Lptn could be detected in the supernatants from untransfected DC, DCLacZ and H22 cells.

Consistent with ELISA results, only the supernatant from DCLptn was positive for chemotaxis assay (chemotaxis index = 3.2 ± 0.15), but from DC, DCLacZ, H22 groups was negative. The results indicate that recombinant Lptn secreted from DCLptn had an effective biological activity.

Recognition and characterization of H22 and DCLptn fusion

Fusion was examined by confocal microscopy (Figure 2) and flow cytometry (Figure 3). The fusion cells were yellow under confocal microscope. The fusion efficiency assayed by FACS was 15%-22%.

FACS analysis showed that DCs encoding lymphotactin were positive for H2-K^d, I-A^d, CD80, CD86, CD40, CD54. However, H22 cells expressed a moderate level of I-Ad. The expression levels of H-2K^d, CD80, CD86, CD40 and CD54 were almost negative. Hybrid DCLptn/H22 cells acquired the phenotypes of both DCLptn and H22 cells.

Enhancement of Th1 cytokine production and CD25 expression

Flow cytometry showed that a very high CD25 expression was observed in T lymphocytes generated in autologous mixed lymphocyte reaction with DCLptn/H22 fusions (58.23% ± 11.65%) when compared to T cells either cultured with DCLptn cells (39.12% ± 12. 35%), H22 (10.78% ± 5. 46%), DC/H22 cells (41.55% ± 12.82%), or DCLptn+H22 cells (43.03% ± 10.52%). By *in vitro* cytokine release assay, significantly higher concentrations of IFN-γ and IL-2 were noted in supernatants of DCLptn/H22 co-cultured with T cells compared to those of DCLptn, DCLptn + H22, DC/H22 or H22 co-cultured with T cells. No difference was noted between concentrations of IL-4 or IL-10 in supernatants of all groups (Table 1).

Elicitation of tumor-reactive CTLs by fusion of DCs with H22 cells

Cytotoxic assay revealed that T cells derived from

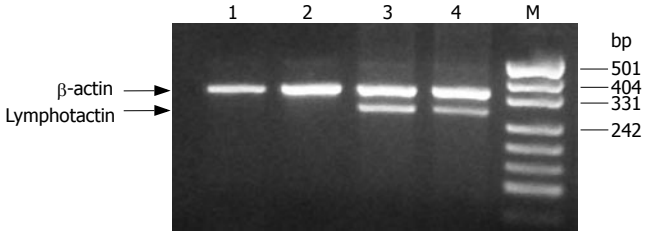

Figure 1 RT-PCR analysis of lymphotactin gene expression in DC, H22, DCLptn, H22Lptn (lanes 1-4). The data shown are representative of three separate analyses for each cell population. M: Marker.

DCLptn/H22 group possessed an extremely higher anti-tumor activity than those derived from DCLptn, H22, DCLptn + H22, DC/H22 groups. Although there were no differences among DCLptn, DCLptn + H22 and DC/H22 groups, the anti-tumor activity of DCLptn, DCLptn + H22 and DC/H22 groups was remarkably higher than that of H22 groups (Figure 4).

DISCUSSION

$CD8^+$ T cells are critical components in immune responses to tumors and can differentiate into cytotoxic T lymphocytes and acquire the ability to lyse tumor antigen expressing cells. Activation of $CD8^+$ T cells requires two steps[18-20]: presentation of antigenic peptides on professional antigen presenting cells and helper function provided by $CD4^+$ T cells *via* Th1/Th2 cytokines. When DCs and HCC cells are fused, antigens are processed and displayed on the cell surface through MHC class I pathway which stimulates $CD8^+$ T cells, and some antigens may be displayed by MHC class Ⅱ molecules, which stimulate $CD4^+$ T cells. On the other hand, mature DCs express MHC Ⅰ, MHC Ⅱ and co-stimulatory molecules that provide necessary signals for the stimulation of naïve T cells[21,22]. Upon stimulation, proliferating $CD4^+$ T lymphocytes differentiate along the Th1 pathway, resulting in increased IFN-γ and IL-2 production, contributing to the activation of tumor-specific CTLs and enhancing the cytotoxic effect. Evidence from cytokine release assays indicates that in cultures with proliferating lymphocytes, the production and secretion of Th1-associated cytokines (IFN-γ, IL-2) but not Th2-associated cytokines (IL-4, IL-10) are increased. In our study, the fusion groups had a higher CTL activity than H22 group.

Activation of lymphocytes is a dynamic, multistep process. Although MHC and costimulatory molecules are critical for successful T-cell activation, signals that regulate this process have not been fully elucidated. It is believed that chemokines are an essential mediator. Migration of DCs to the sites of inflammation where they capture antigens and subsequently migrate to the local lymph nodes is regulated by the expression of different chemokines and their receptors[23,24]. Lymphotactin as a C chemokine produced mainly by T and nature killer (NK) cells, is a chemoattractant both *in vitro* and *in vivo*[25-28]. In our study, DCs and H22 cells did not express Lptn, and the Lptn gene-modified hybridima had a stronger CTL activity

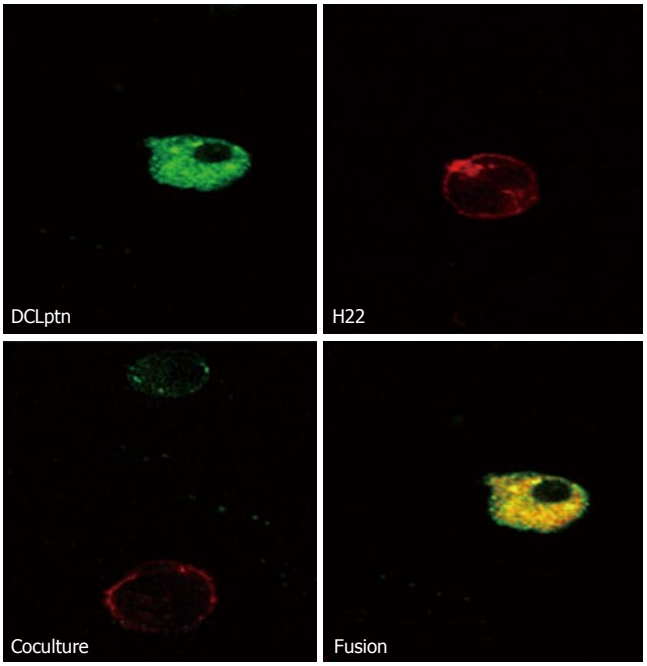

Figure 2 Confocal micrography of DCLptn/H22 fusion cells.

and a higher Th1 cytokine production, suggesting that Lptn modification can improve preferential chemotaxis of hybridoma on T cells and consequently optimize the microenvironment of antigen presentation to T cells.

CD25, α-chain of the IL-2 receptor, is expressed in the early to moderate phase after T-cell activation, the clonal proliferation of activated T cells depends on the expression of this receptor and resting lymphocytes do not express CD25[29,30]. Therefore, CD25 expression is commonly used as a marker for T cell activation. Quantification of surface IL-2 receptor expression on activated lymphocytes by flow cytometry after *in vitro* stimulation with specific antigens is useful in measuring cellular immunity. In the present study, we used this method to assess the lymphotactin gene-modified hybridoma's stimulation on co-cultured T cells. By using this method, we were able to study the effect of stimulation on a heterogeneous cell population without the risk of selective depletion of cells, to exclude non-specific stimulation due to the separation, and to express CD25 at the early to moderate (24-48 h) phase of mixed lymphocyte reaction, thus shortening the co-culture time and keeping the viability of T cells.

In conclusion, lymphotactin gene-modified dendritoma induces potent T-cell proliferation and strong CTL reaction against allogenic HCC cells. Immunization-engineered fusion hybrid vaccine is an attractive strategy in prevention and treatment of cancer metastases.

COMMENTS

Background

Despite recent advances in surgical technique and radio- and chemotherapy, the prognosis of patients with malignant tumors remains dismal. The resistance of these tumors to conventional treatment may stem from their well-documented ability to exert local and systemic immunosuppressive effects. Therefore, alternative treatments are required.

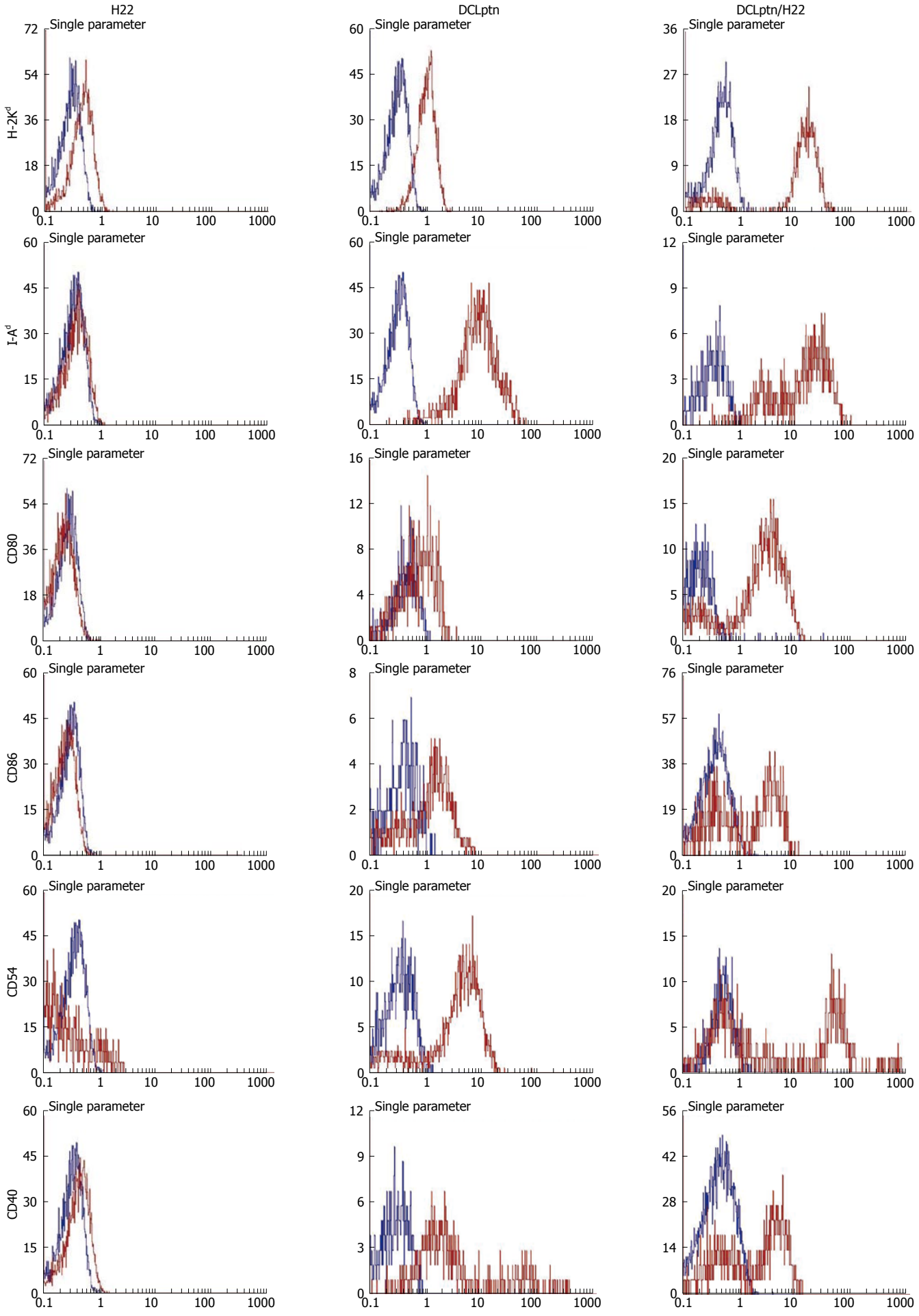

Figure 3 FACS analysis of the phenotypes of H22, DCLptn and DCLptn/H22 fusion cells.

Table 1 Cytokine concentration in MLR supernatants after different cell population stimulation (pg/mL, mean ± SD)

	IFN-γ	IL-2	IL-10	IL-4
A (H22)	510.3 ± 9.32	39.7 ± 2.72	91.48 ± 1.59	58.64 ± 0.4
B (DCLptn)	1015.51 ± 7.2[f]	88.47 ± 3.17[f]	96.20 ± 1.27	55.89 ± 2.95
C (DCLptn+H22)	999.64 ± 11.86[f]	82.39 ± 3.02[f]	97.33 ± 2.23	59.78 ± 1.21
D (DC/H22)	992.45 ± 10.16[d,f]	93.28 ± 0.91[d,f]	131.94 ± 0.32[d]	98.71 ± 2.14[d]
E (DCLptn/H22)	1886.08 ± 56.75[b]	170.12 ± 2.11[b]	217.13 ± 1.91[b]	167.58 ± 0.94[b]

[b]$P < 0.01$ *vs* A, B, C, D; [d]$P < 0.01$ *vs* A, B, C; [f]$P < 0.01$ *vs* A.

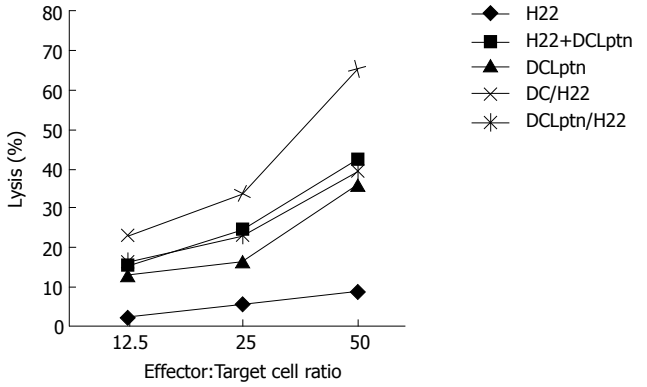

Figure 4 Stimulation of anti-tumor CTLs by DCLptn/H22 cells.

Research frontiers

Dendritic cells are the most potent APC for inducing an antigen-specific CTL response. This property, coupled with the fact that it is now possible to generate, *ex vivo*, a large number of functional dendritic cells from a patient's peripheral blood monocytes or CD34 haemopoietic stem cells, have led to a considerable interest in use of dendritic cell vaccines as a means to induce antitumour immunity. Various strategies have been developed to introduce tumor specific antigens into DCs and thereby to generate cytotoxic T lymphocyte (CTL) responses against malignant cells. One of the important approaches to the induction of primary antitumor immunity is through the generation of tumor cell and DC fusion.

Innovations and breakthrough

Although some effective results have been obtained by vaccinating mice with fusion of DCs and other tumor-cell types, it still remains a challenge. Several parameters must be optimized in order to maximize the efficacy of immunotherapy for dendritoma. In the present study, the authors have found that after Lptn gene modification, activated T cells can acquire more tumor antigens from DCLptn/H22 and have a stronger cytotoxicity to target cells.

Applications

This may be an attractive strategy in prevention and treatment of cancer metastases.

Terminology

Dendritoma: fusion formed by dendritic cells and carcinoma cells.

Peer review

This paper investigated the *in vitro* activation of cytotoxic T lymphocytes by fusion of mouse hepatocellular carcinoma (HCC) cells and dendritic cells modified by transfection of the lymphotactin gene. The authors conclude that lymphotactin modifies dendritoma and induces T cell proliferation and strong reaction of cytotoxic lymphocytes against allogenic HCC cells. These results are of certain interest.

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CASE REPORT

Poorly differentiated carcinoma of the rectum with aberrant immunophenotype: A case report

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Key words: Rectal adenocarcinoma; c-kit immunoreactivity; Treatment

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Abstract

We report a case of a poorly differentiated epithelial tumour of the rectum with a highly pleomorphic morphology and an aberrant immunophenotype, including the expression of epithelial markers, the focal parameter of neuroendocrine differentiation, and the unexpected detection of CD-117 overexpression. A 69-year-old man was admitted to our clinic complaining of rectal bleeding and weight loss. Colonoscopy showed an ulcerative bleeding mass located about 8 cm from the anal verge. Abdominal and pelvis CT scans demonstrated a large low-density lesion with extracanalicular growth from the middle rectum, with local lymph-node spread, and without tumour infiltration of other pelvic organs, or evidence of distant intra-abdominal spread. The patient underwent a low anterior resection for rectal cancer together with wide resection of lymph nodes. In immunohistochemical analysis, pankeratin and Epithelial Membrane Antigen (EMA) immunolabeling proved the epithelial nature of the tumor cells. Chromogranin A and Leukocyte Common Antigen (LCA) were negative, whereas CD-56 expression was scanty and Neuron Specific Enolase (NSA) was heavily and diffusely expressed. Ki67 immunoexpression was particularly increased. Interestingly, the intense c-kit immunoreactivity (100%) was a common feature. The above phenotypic and immunohistochemical profile was consistent with an anaplastic carcinoma of the large intestine, with focal neuroendocrine differentiation and diffuse immunoreactivity to c-kit protein. Given the resistance of this tumor to conventional chemotherapy and radiation, the incidence of the c-kit alteration may represent a novel approach to a gene-directed treatment using a c-kit inhibitor (STI571) similar to that which has been proposed in GISTs.

INTRODUCTION

We report a case of a poorly differentiated epithelial tumour of the rectum with a highly pleomorphic morphology and an aberrant immunophenotype, including the expression of epithelial markers, the focal parameter of neuroendocrine differentiation, and the unexpected detection of CD-117 overexpression.

CASE REPORT

A 69-year-old man was admitted to our clinic complaining of rectal bleeding for 2 mo (two episodes of massive rectal bleeding) and weight loss of 5 kg in 4 mo. His past medical history was negative for any surgical procedure or chronic disease, and his family history was also free. He denied any change in bowel habits, urinary urgency, or any other symptoms. Digital examination was normal but proctosigmoidoscopy showed an ulcerative mass bulging over the right rectal wall, and the fecal examination was positive for blood.

Laboratory tests of the peripheral blood revealed microcytic hypochromic anemia (hemoglobin, 11.7 g/dL and hematocrit, 26.6%). The serum levels of carcinoembrionic antigen (CEA), alpha-fetoprotein AFP, and CA19-9 were within normal ranges. Prostate-specific antigen (PSA) was also within the normal range (0.7 ng/dL, PSA free, 0.16 mg/dL).

Colonoscopy showed an ulcerative bleeding mass that was located about 8 cm from the anal verge. An additional abnormality revealed by colonoscopy was the existence of five small polyps along the rest of the colon. Abdominal and pelvis CT scans demonstrated a large low-density lesion with extracanalicular growth from the middle

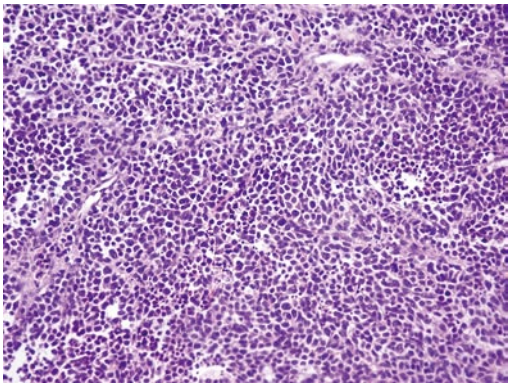

Figure 1 Histological appearance of the colorectal adenocarcinoma (HE, × 20).

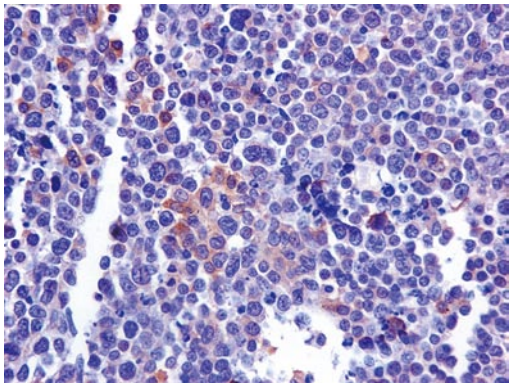

Figure 2 Scanty CD-56 immunohistochemical expression by tumor cells (× 40).

rectum, with local lymph-node spread, and without tumour infiltration of other pelvic organs, or evidence of distant intra-abdominal spread. No metastatic nodules were found in the lung and the liver by diagnostic imaging procedures. The patient underwent a low anterior resection for rectal cancer with a circular stapled low, end-to-end colorectal anastomosis (indicated for tumours situated 6-9 cm above the anal verge), together with wide resection of lymph nodes.

On gross examination, the 14-cm rectosigmoidal surgical specimen manifested as an ulcerative tumor that measured 5 cm in its larger diameter, located 2-5 cm from the distal resection margin.

Under microscopy, the tumor was composed of irregular sheets and scattered tumor cells (Figure 1) with markedly pleomorphic nuclei and prominent nuclei, including giant or multinucleated cell types. The tumor was found to infiltrate the submucosa, the muscularis propria, and the perirectal adipose tissue. Nodal metastasis was found in 2/22 lymph nodes examined.

In immunohistochemical analysis, pankeratin and Epithelial Membrane Antigen (EMA) immunolabeling proved the epithelial nature of the tumor cells. Chromogranin A and Leukocyte Common Antigen (LCA) were negative, whereas CD-56 expression was scanty (Figure 2), and Neuron Specific Enolase (NSA) was heavily and diffusely expressed. Ki67 immunoexpression was particularly increased. Interestingly, the intense c-kit immunoreactivity (100%) was a common feature (Figure 3A and B). The above phenotypic and immunohistochemical profile was consistent with an anaplastic carcinoma of the large intestine, with focal neuroendocrine differentiation and diffuse immunoreactivity to c-kit protein.

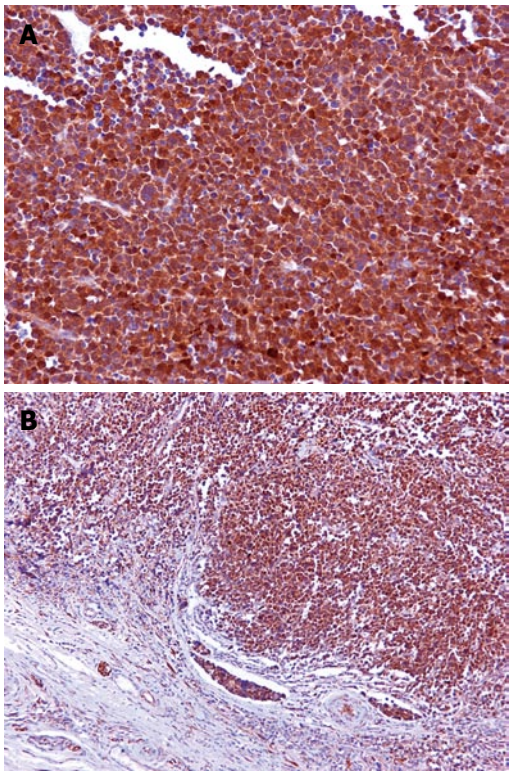

Figure 3 **A**: Intense c-kit immunolabeling (× 20); **B**: intense nuclear cytoplasmic immunolabeling for c-kit protein (× 10).

DISCUSSION

c-kit protein, a 145-kDa tyrosine kinase with oncogenic properties is a transmembrane receptor growth factor known as a stem cell factor (SCF). It is encoded by the c-kit proto-oncogene located on chromosome 4q11-q12[1]. Activation of c-kit by its SCF ligand leads to dimerization of the receptor. The latter activates further signalling cascades that control cell proliferation, adhesion and differentiation[2].

CD-117 is a functionally important protein in hematopoietic stem cells, mast cells, germ cells, some epithelial cells and in Cajal cells. Parenthetically, Cajal cells are known to originate from common intestinal mesenchymal precursor cells[2-5].

Several studies have identified the presence of a c-kit malignant mutation in over half of gastrointestinal stromal tumors (GISTs), as well as in other human tumors, including germ cell tumors, neuroblastoma, melanoma, ovarian carcinoma and breast carcinoma[6-14]. Interestingly, overexpression of c-kit has been found to affect proliferation in human neural, lung, breast, colorectal, skin and prostatic tumors[15].

On the basis of an immunohistochemical study of c-kit expression in 126 colorectal carcinomas, only two

(1.6%) poorly differentiated carcinomas presented with aberrant c-kit positivity, which implies the role of c-kit in tumor progression[16]. Although the functional role of mutated c-kit kinase activity is not fully understood, it seems that in breast, thyroid and ovarian cancer, the malignant transformation seems to correlate with loss of c-kit protein expression[17]. However, Bellon *et al*[12] have reported overexpression of c-kit in human colorectal cancer, and have suggested that c-kit activation is critical for growth, survival, migration and invasive potentional of DLD-1 colon carcinoma cells. Of interest, only 1.6% of colorectal cancers show high cytoplasmic c-kit staining, a fact that is not related definitely to tumorigenesis[16]. Immunohistochemical expression of c-kit protein is a rare event in poorly differentiated carcinomas[16,17].

In the study by Akintola-Ogunremi *et al*[17], who studied 66 cases of primary colorectal neuroendocrine carcinoma, the prognosis did not appear to differ between kit-positive and kit-negative cases. In the view of the limited number of reports in the literature and the lack of follow-up data, c-kit overexpression cannot provide any evidence regarding the biological behavior of the tumor currently described. However, further follow-up, together with c-kit gene mutational analysis may alter the prognostic value of c-kit positivity in these highly aggressive malignancies of the colon. Thus, the immunohistochemical CD-117 alteration in poorly differentiated carcinoma of the rectum remains to be elucidated.

Given the resistance of this tumor to conventional chemotherapy and radiation[18,19], the incidence of the c-kit alteration may represent a novel approach to a gene-directed treatment using a c-kit inhibitor (STI571) similar to that which has been proposed in GISTs[20]. According to the literature, STI571 may inhibit the *in vitro* growth of colorectal carcinoma cell lines, although it has not been tested so far for the treatment of colorectal carcinoma[20].

A long term study of c-kit protein expression in poorly differentiated malignancies of colon may be warranted, although c-kit overexpression can not guarantee tumor response. Thus, a thorough genetic investigation of colorectal malignancy may determine the eligibility of STI571 regimen for potential targeted therapy.

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CASE REPORT

Antegrade bowel intussusception after remote Whipple and Puestow procedures for treatment of pancreas divisum

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Abstract

To date, antegrade intussusception involving a Roux-en-Y reconstruction has been reported only once. We report a case of acute bowel obstruction due to an intussusception involving two Roux-en-Y limbs in a 40-year-old woman with a history of chronic pancreatitis due to pancreas divisum. Four years preceding this event, the patient had undergone a Whipple procedure, and three years prior to that, a Puestow operation. The patient was successfully treated with bowel resection and a side-to-side anastomosis between the most distal aspect of the bowel and the most distal Roux-en-Y reconstruction, which preserved both Roux-en-Y reconstructions.

Key words: Whipple procedure; Puestow procedure; Pancreas divisum; Intussusception; Bowel obstruction

Gigena M, Villar HV, Knowles NG, Cunningham JT, Outwater EK, Leon LR Jr. Antegrade bowel intussusception after remote Whipple and Puestow procedures for treatment of pancreas divisum. *World J Gastroenterol* 2007; 13(44): 5954-5956

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INTRODUCTION

It is a well known fact that intussusception is most often seen in children[1]. Intussusception in adults however is relatively rare, with about 17% of intussusception cases in large reported series having occurred in adults[1]. Surgical sutures or staples along an anastomosis are, among other factors, well-known lead points for the development of intussusception, therefore making abdominal surgical interventions recognized risk factors for the occurrence of this complication[2-6]. Intestinal tract reconstructive surgery involving the pancreas however, has been very rarely linked to the development of intussusception[7]. We report the case of a middle-aged woman who developed intussusception after two major operations that were remotely performed for the therapy of symptomatic pancreas divisum. A brief discussion of the available literature is also presented.

CASE REPORT

A 40-year-old woman presented with abdominal pain, nausea and vomiting of 24 h duration. She was afebrile and normotensive but had tachycardia. Her upper abdomen was visibly distended and a palpable epigastric mass could be felt. The abdomen was severely tender to palpation and peritoneal signs were elicited. Her past history was significant for pancreas divisum and chronic pancreatitis. Four years prior, she underwent a Whipple procedure as therapy for her pancreatic abnormalities. This required surgical revision 1 year later with a Puestow operation, due to stricture of the previously performed pancreatico-intestinal anastomosis. Ever since, she experienced intermittent abdominal pain, for which she was prescribed strong analgesics, with only partial symptomatic relief. Her white blood cell count was 17 000 cells/mm^3. A computed tomography (CT) scan of the abdomen was obtained, which demonstrated jejunal intussusception, with findings suggesting bowel ischemia (Figure 1).

After fluid resuscitation, the patient was subjected to an exploratory laparotomy. A small amount of ascites was encountered. Two loops of dilated small bowel were found inferior to the transverse mesocolon, each measuring about 10 cm in maximal diameter. These loops were identified to be part of the previously performed Roux-en-Y and Puestow procedures, going towards the stomach, bile duct and the pancreatico-jejunostomy reconstruction. Upon further exploration, an intussusception just distal to the most distal Roux-en-Y connection was found, and about 30 cm of non-perforated necrotic small bowel was identified. The intussusception occurred in an antegrade fashion, which obstructed both Roux-en-Y reconstructions. With care, the intussuscepted intestine was reduced. The necrotic bowel was then resected, and a side-to-side anastomosis between the most distal aspect of the bowel and the Roux-en-Y reconstruction that was directed towards the Puestow procedure was performed.

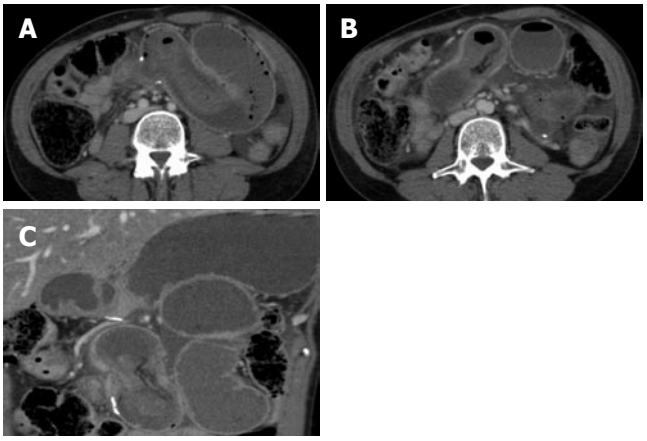

Figure 1 Axial (**A** and **B**) and coronal (**C**) CT images of the abdomen following intravenous contrast administration, which show large dilated loops of small bowel proximal to the intussusception. The intussuscepted bowel entered the more distal jejunum *via* the jejunal anastomotic site, which is evident due to the presence of surgical clips.

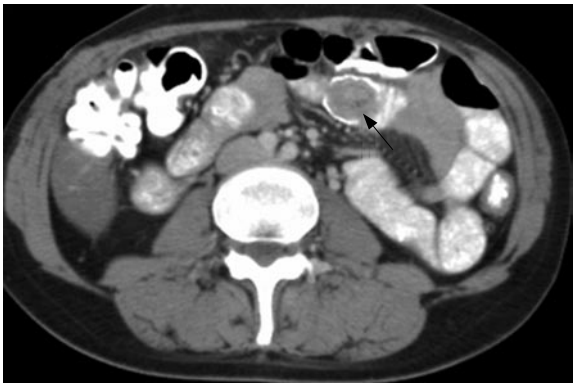

Figure 2 Abdominal CT scan from prior hospital visits, which reveals milder bowel intussusception prior to the patient's last admission.

Due to the massive mesenteric vascular engorgement caused by the intussusception, there was an area of bleeding emanating from a bowel mesentery tear. This was localized and controlled. The abdomen was lavaged and closed. Postoperatively, the patient developed clinical evidence of abdominal compartment syndrome and required emergent re-exploration and blood transfusion. The mesenteric tear was again found to be the source of massive bleeding, and was repaired with additional stitches. Temporary skin closure of the abdomen was performed. Final closure was performed 3 d after the first intervention, and she was discharged without complications 8 d later.

DISCUSSION

Pancreas divisum is an anomaly of the pancreatic ducts, which represents the most common congenital variant of the pancreas. It results from the absence of embryological fusion of the dorsal and ventral pancreatic ducts, each keeping their drainage autonomy[8]. The correlation of this abnormality with pancreatic disease is very controversial[9]. Several techniques have been suggested for therapy, including endoscopic papillotomy, open surgical accessory sphincteroplasty, or a Puestow procedure[10]. As a result of the underlying duct anomalies and significant pancreatic head changes, some have suggested treatment with duodenum-preserving pancreatic head resection (Beger's pancreatectomy)[10]. With good patient selection, the outcome of surgical therapy has been shown to be acceptable.

Our patient underwent a Whipple procedure for chronic pancreatitis, which did not achieve symptomatic relief. This was likely due to stenotic involvement of the entire pancreatic duct, and not only the head portion, as well as due to a stricture at the pancreatico-intestinal anastomosis. This was recorded in the patient's old medical records. In consequence, a Puestow operation was subsequently performed, which resulted in symptomatic improvement but incomplete relief. The latter procedure would have likely been a better first modality of therapy for this patient upon her initial presentation, together with a papillotomy of the minor papilla. However, endoscopic retrograde cholangiopancreatography images were not available to us, and it is therefore impossible to give an accurate opinion about her initial treatment.

The chronic nature of our patient's symptoms made her diagnosis challenging. This was due to the fact that she had recurrent symptoms of abdominal pain, nausea and vomiting after both interventions, and that she required large doses of analgesics and antidepressants due to chronic pain. In fact, previously performed CT scans revealed milder degrees of small bowel intussusception in prior hospital visits (Figure 2), which were thought to represent transient short bowel segment intussusceptions.

It has been suggested that altered intestinal motility may contribute to the development of intussusception[2]. In fact, this complication may be an extreme form of the so-called Roux-en-Y stasis syndrome[3]. It has been shown that the myoelectric activity of the Roux limb is often dysfunctional, split and retrograde, and of high amplitude (> 120 mmHg). Therefore it is possible that the intussusception seen in our patient was the result of severe disruption of the normal pacemaker activity in the intestines[3]. This is even more likely given the fact that we did not identify any intraluminal, extraluminal or intramural lesions. Her current presentation with necrotic bowel did not allow us to perform further imaging studies (i.e., small bowel follow through or gastric emptying studies) to demonstrate altered motility and peristaltic motion, and, rather, mandated emergent exploration.

Cases of small bowel intussusception in adults without a lead point have rarely been reported. They are most often seen after gastric bypass is performed for morbid obesity[4,5], but also have been reported exceptionally after biliary reconstruction for choledochal cysts[6], or associated with *Vibrio* infection in a patient with diabetic ketoacidosis[11]. Intussusception occurring after pancreatic duct reconstruction is extremely rare. It was reported for the first time after a pancreatico-jejunostomy in 2003[7]. The latter case reported retrograde intussusception of the efferent limb into the anastomosis of a revised Roux-en-Y bypass of the pancreas, similar to our case. Our patient represents the second reported case of an

antegrade intussusception that occurred after pancreatic reconstruction. The retrograde case that was reported by Whipple and colleagues[7], occurred after a Roux-en-Y revision for an antegrade intussusception after a Puestow procedure performed for chronic pancreatitis.

A lead point is identified in approximately 80% of intussusception cases[7]. In the current case, we were not able to identify a lead point. Interestingly, neither was this noted in the case reported by Whipple *et al*[7].

Our patient unfortunately developed abdominal compartment syndrome due to massive hemoperitoneum. The massive intestinal dilatation accounted for the friability of the bowel mesentery, which, together with an elevated venous pressure caused by blood flow obstruction in the caval-mesenteric veins, due to the mass effect produced by the bowel obstruction, may explain the large amount of bleeding. Permanent abdominal closure after our second intervention was precluded because of bowel edema and disseminated intravascular coagulation after massive resuscitation, due to the large amount of blood loss.

In conclusion, antegrade intussusception in adults after pancreatic duct reconstruction is extremely rare. Our case represents the second report in the literature of such an occurrence. This patient had previous episodes of abdominal pain, nausea and vomiting, which suggests that altered intestinal motility may have contributed to her current presentation. Bowel intussusception should be always considered in cases of small bowel obstruction in adults after pancreatic reconstruction.

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NEWS

Lian-Sheng Ma, Editor-in-Chief of *WJG*, warmly meets Professor Hugh J Freeman from the University of British Columbia

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Abstract

Lian-Sheng Ma, Editor-in-Chief of *World Journal of Gastroenterology* (*WJG*), warmly met Professor Hugh J Freeman from the University of British Columbia at Peninsula Hotel in Beijing on August 28, 2007. Professor Hugh J Freeman gave much helpful advice toward the further development of *WJG*. He will serve as series editor for a new column called OBSERVER which will start in *WJG* in 2008.

Chang YD. Lian-Sheng Ma, Editor-in-Chief of *WJG*, warmly meets Professor Hugh J Freeman from the University of British Columbia. *World J Gastroenterol* 2007; 13(44): 5957

http://www.wjgnet.com/1007-9327/13/5957.asp

Professor Lian-Sheng Ma (left), Editor-in-Chief of *WJG*, and Dr. You-De Chang (right) warmly met Professor Hugh J Freeman (middle) at Peninsula Hotel in Beijing. Photograph taken by Mrs. Sally Freeman.

Professor Lian-Sheng Ma, Editor-in-Chief of *World Journal of Gastroenterology* (*WJG*) warmly met Professor Hugh J Freeman, a highly respectable gastroenterologist from the Department of Medicine, University of British Columbia, and his wife Mrs. Sally Freeman during their visit to *WJG* at Peninsula Hotel in Beijing on August 28, 2007. Both sides achieved very fruitful talks and reached a couple of common viewpoints related to future development strategy and management of *WJG*.

Professor Ma gave a detailed introduction to the strategies of both fast peer review and online free access currently taken by *WJG*. "Both fast peer review and online free access are very beneficial and competitive." replied Hugh J Freeman, "It is really just like having a lesson through reading the comments affiliated at the end of an article." He also suggested that *WJG* openly add the names of the peer reviewers at the end of the affiliated comments to make the science communities of authors, reviewers and readers more active and real. "These three aspects have played important roles in ensuring the quality of articles and increasing the public access to *WJG*." he added.

Professor Freeman encouraged with confidence the authors-created, innovation-orientated and readers-benefited publishing system currently conducted by *WJG* with little commercial evolvement. "Over commercial evolvement sometimes misleads the path a journal takes and weakens the decisions a journal makes." Freeman pointed out.

As the second important topic of their talks, Professor Ma invited Professor Freeman to be Associate Editor-in-Chief for a unique column called OBSERVER which will start in *WJG* in 2008. Freeman kindly accepted the invitation. The OBSERVER column will serve as a forum for both gastroenterologists and hepatologists worldwide. Professor Freeman will periodically invite a set of experts from specific research fields to discuss a series of hot topics covering the progress made in both gastroenterology and hepatology, and the challenging questions currently faced by gastroenterologists and hepatologists as well as the possible ideas, ways and techniques to answer these questions. The OBSERVER is an invited editorial for free of publication. For more information, please do not hesitate to contact Professor Freeman at hugfree@shaw.ca and Science Editor Dr. You-De Chang at y.d.chang@wjgnet.com.

On behalf of both Professor Hugh J Freeman and the upcoming OBSERVER column, the *WJG* staff sincerely thank all editorial members, authors and readers from around the world and warmly welcome your coming submissions.

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Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Inflammatory Bowel Diseases 2007
1-3 March 2007
Innsbruck
ibd2007@come-innsbruck.at
www.come-innsbruck.at/events/ibd2007/default.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting

Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
18-22 April 2007
Paris Hotel and Casino, Las Vegas, Nevada
www.sages.org/07program/index.php

Meeting Falk Symposium 159: IBD 2007-Achievements in Research and Clinical Practice
4-5 May 2007
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symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
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fca@netvisao.pt

Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in Gastroenterology
15-16 June 2007
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Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
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Meeting 9th World Congress on Gastrointestinal Cancer
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Meeting 15th International Congress of the European Association for Endoscopic Surgery
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info@eaes-eur.org
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Meeting 39th Meeting of the European Pancreatic Club
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Meeting X X th International Workshop on Heliobacter and related bacteria in cronic degistive inflammation
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Meeting Falk Symposium 161: Future Perspectives in Gastroenterology
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Author(s) and editor(s)

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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