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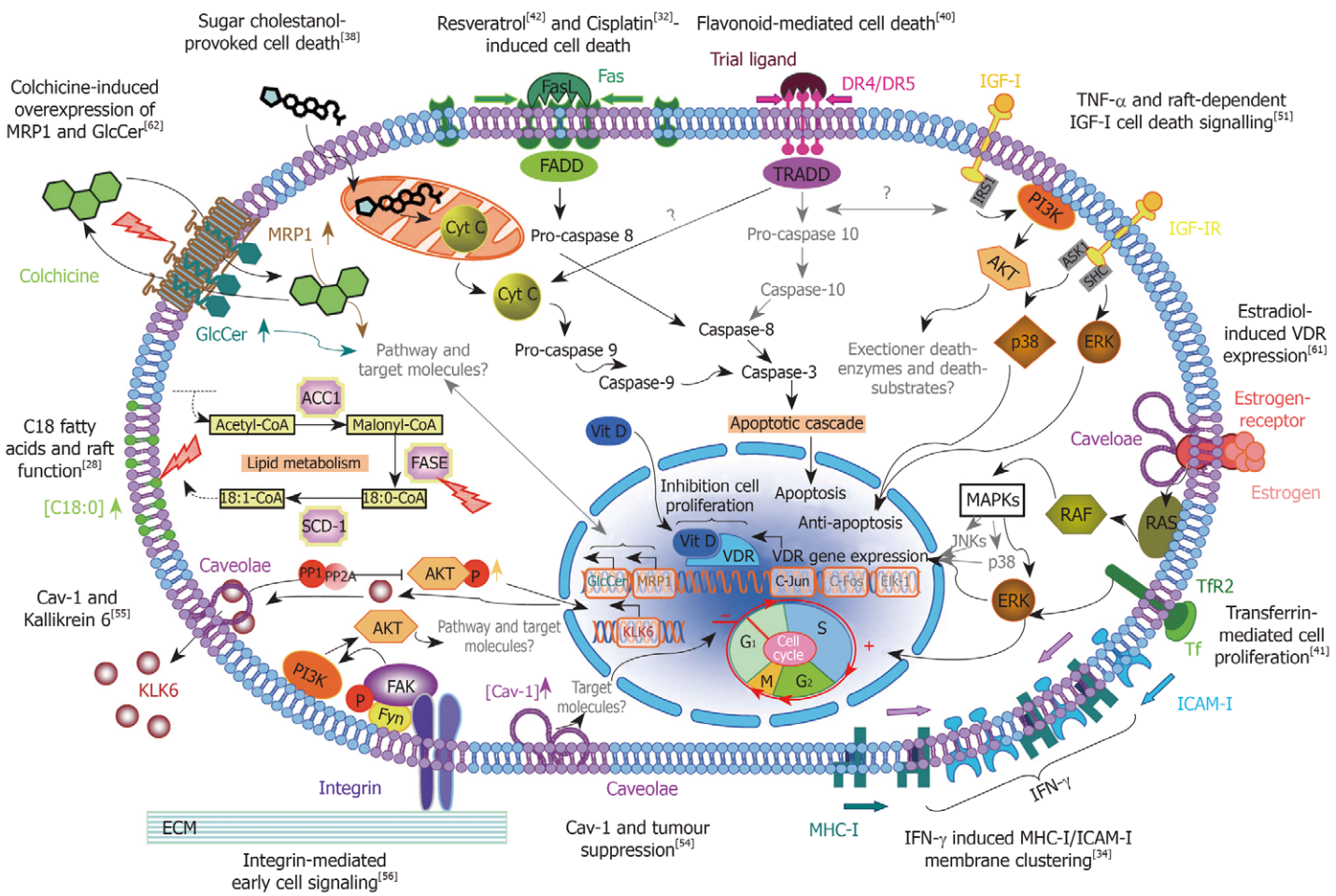

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EDITORIAL

Multifaceted nature of membrane microdomains in colorectal cancer

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Abstract

Membrane microdomains or lipid rafts are known to be highly dynamic and to act as selective signal transduction mediators that facilitate interactions between the cell's external and internal environments. Lipid rafts play an important mediating role in the biology of cancer: they have been found in almost all existing experimental cancer models, including colorectal cancer (CRC), and play key regulatory roles in cell migration, metastasis, cell survival and tumor progression. This paper explores the current state of knowledge in this field by highlighting some of the pioneering and recent lipid raft studies performed on different CRC cell lines and human tissue samples. From this literature review, it becomes clear that membrane microdomains appear to be implicated in all key intracellular signaling pathways for lipid metabolism, drug resistance, cell adhesion, cell death, cell proliferation and many other processes in CRC. All signal transduction pathways seem to originate directly from those peculiar lipid islands, thereby orchestrating the colon cancer cells' state and fate. As confirmed by recent animal and preclinical studies in different CRC models, continuing to unravel the structure and function of lipid rafts - including their associated complex signaling pathways - will likely bring us one step closer to better monitoring and treating of colon cancer patients.

Key words: Actin; Caveolae; Cytoskeleton; Combined imaging; Detergent-resistant membranes; Drug targeting; Electron microscopy; Lipid domains; Membrane rafts; Prognosis; Staging; Tomography; Lipid-mediated therapy

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INTRODUCTION

In recent years, high-speed multidimensional live-cell confocal laser imaging[1], often combined with combinatorial labeling approaches[2] and/or subsequent correlative electron microscopy studies[3], has allowed high-throughput studies that add missing pieces of temporal and spatial resolution and detail to the cell membrane puzzle. At the nanometer length scale, semi-automated transmission electron tomography (TET) has enabled generation of accurate three-dimensional models of the fine architecture of the cell membrane and its associated proteins[4,5]. Meanwhile, advances in the high-throughput analyses of chemical and molecular biology have allowed researchers to catalogue the lipidomics of the cell with hitherto unseen sensitivity[6,7], complementing recent computer simulations of lipid membranes[8,9]. Over the last decade, this

progress in analytical chemistry and in rapid microscopy-based imaging has underpinned the exponential growth in our understanding of the dynamic and multifaceted role of the cell membrane in various physiological processes.

The cell membrane is unquestionably one of the most studied subcellular components in the modern era of cell biology[10-12]. This is not simply because it is an attractive target for drug designers[13], but also because it acts as a selective protector of the interior of the cell[14]. Key regulatory roles for the cell membrane have been demonstrated in cell homeostasis and differentiation, cell death and cell survival pathways, inter- and intracellular signaling, cell development and movement, and trans- and intercellular transport mechanisms[15,16]. Of special interest are unique lipid domains within the cell membrane that have been shown to be involved, directly or indirectly, in such lipid-mediated cell regulation. These small domains, also known as lipid rafts, were initially described by Palade through the use of electron microscopy[17] and later carefully identified by Simons *et al*[18] through a combined biochemistry and imaging approach. These rafts lie as discrete patches, known as detergent-resistant membrane structures, in the plasma membrane of cells and are rich in sphingolipids and cholesterol[18,19]. The fatty acid chains of lipids within these rafts tend to be tightly packed, creating ordered lipid domains that float in a sea of poorly-ordered lipids within the membrane[20]. Lipid rafts are highly dynamic and temperature sensitive, are able to form large clusters and to interact with the cell's internal molecular and structural compartments[21,22]. From recent studies, it is becoming ever more evident that rafts act as highly dynamic and selective guardians between the cell's external and internal worlds, which makes researchers view them as important structural and molecular targets for altering cell function and behavior (for a review[23]).

With regard to the biology of cancer, it has been demonstrated that lipid rafts play important mediating roles in cell migration, metastasis, cell survival and tumor progression[24,25]. The literature of the past five years contains over one thousand original research papers that have studied the role of these peculiar lipid islets in cancer. This illustrates their importance and their perceived potential in future cancer cures and/or as markers for tumor staging and, hence, diagnosis and prognosis. This literature also shows the presence of lipid rafts in almost all existing experimental cancer models, *in vitro* and *in vivo*. The presence and role of rafts have also been highlighted within relevant human and clinical settings[26,27], including in colorectal cancer (CRC)[28,29]. Lipid rafts in CRC cells were observed initially in 1998 by Orlandi and Fishman[30] and studied extensively by many others subsequently (see next section). So far, however, no dedicated paper has addressed "raft biology and pathobiology" in CRC. Here, therefore, we carefully review the current state of knowledge by highlighting some of the pioneering and recent raft studies carried out on different CRC cells and tissue samples.

MEMBRANE MICRODOMAINS IN COLORECTAL CANCER CELLS

Researchers have shown, for example, that lipid rafts in CRC cells act as go-betweens for cell death-mediated signaling[31,32], as portals for bioactive compounds[33], and as congregation regions for adhesion proteins and major histocompatibility complex class 1 (MHC-1) molecules[34]. However, despite the abundance of literature available on the biology and pathobiology of lipid rafts in cancer[35], only a few dedicated papers presently address the importance of these membrane domains in the process of CRC. While lipid rafts have been thoroughly described in other malignant tumor models such as cancers of the breast, lung and prostate, this does not imply that we should overlook their important role in the onset and development of CRC (see below). Unraveling their structure and function - including their associated complex signaling pathways - could eventually form the basis for future therapeutic interventions.

Labeling and morphometric imaging approaches

Although lipid rafts have been studied extensively through dedicated labeling and microscopy techniques[36], relatively little is known about their fine structure in CRC cells or tissue at the nanometer scale[37]. The majority of CRC membrane raft studies use immunofluorescence microscopy, often in conjunction with protein blotting and/or flow cytometry. Microscopic identification of lipid rafts in CRC cells is frequently performed by direct labeling against specific molecular components of membrane rafts[32,38]; the classic example is the use of fluorescently-labeled cholera toxin against ganglioside II3-N-acetylneuraminosyl-gangliotetraosylceramide (GM1)[39-41]. Indirect identification approaches are frequently used as well, such as staining against molecular targets or proteins that are supposed to associate with CRC lipid microdomains[42]. However, combinatorial protein blotting studies on isolated raft protein fractions must be an essential part of this approach to unambiguously identify the association of raft-specific proteins with lipid rafts. The different caveolin (Cav) isoforms - predominantly Cav-1 and Cav-2 - are also popular protein targets for raft detection[43,44], but should be used with caution because the degree of Cav expression in cancer cells depends on the differentiation status of the cells and the cancer model studied[35].

Depending on the imaging approach applied, lipid rafts have been reported to range from between 30 and 100 nanometers up to almost one micrometer in size[20,45,46]. We have applied correlative fluorescence and electron microscopy (CFEM) to confirm that the same holds true for CRC cells[37]. CFEM analysis of Caco-2 cells labeled for GM1 allowed the direct observation of fluorescently-labeled lipid rafts by light-optical microscopy and by electron microscopy (Figure 1A). We observed that the smaller lipid rafts could be easily resolved under electron microscopy, but could not be clearly seen by confocal imaging. In some

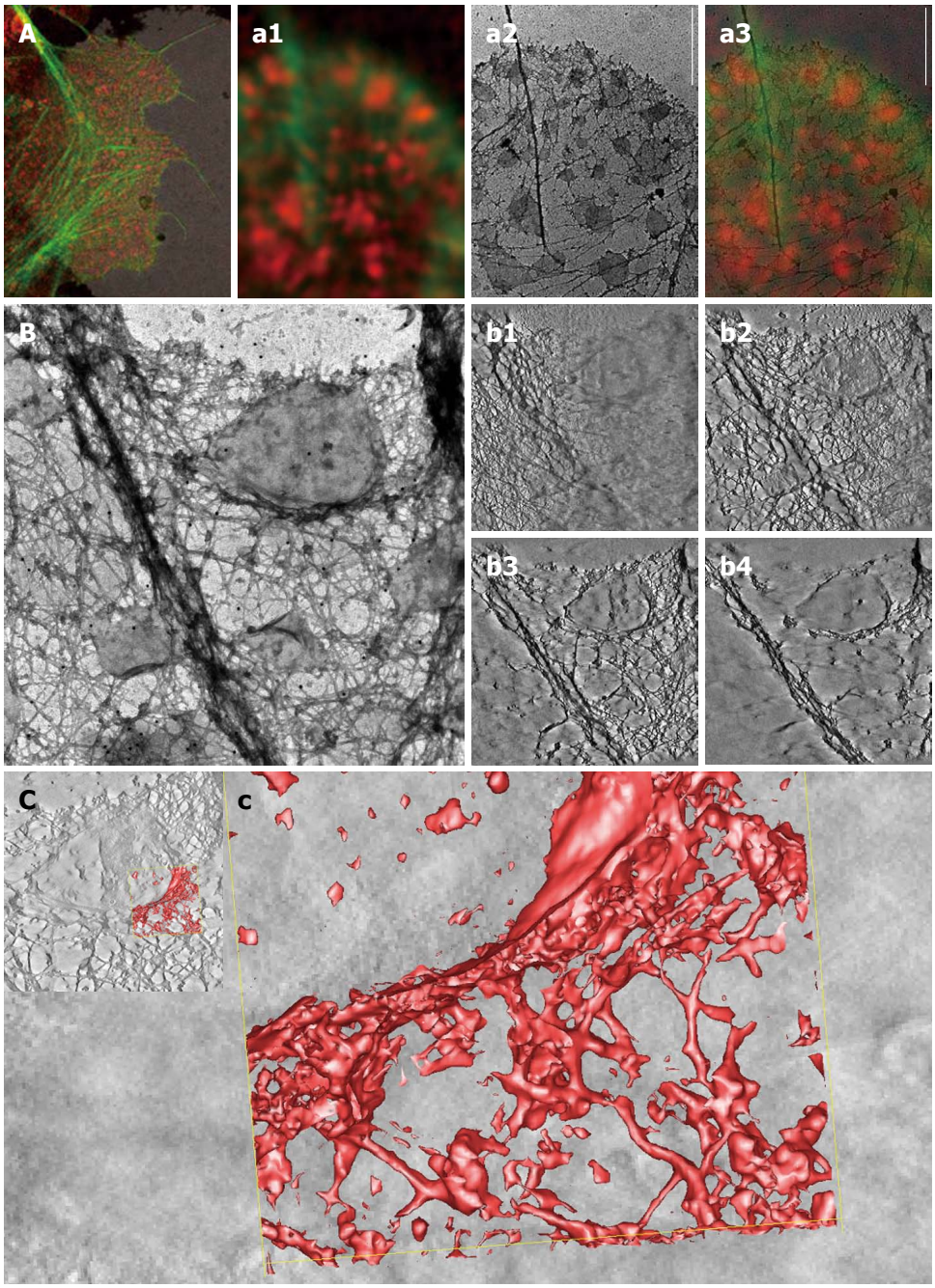

Figure 1 High-resolution imaging of membrane domains in human Caco-2 colorectal cancer cells. These data come from high-resolution correlative fluorescence and transmission electron microscopy (CFEM) studies on whole mounts. Human colorectal cancer (CRC) cells (Caco-2) were cultured on formvar-coated nickel grids and then treated with Triton X-100 in cytoskeleton stabilization buffer, leaving detergent-resistant membranes behind[37]. These membrane fractions were labeled with the membrane raft marker GM1 CTxB-594 (red) and the actin cytoskeleton was stained with phalloidin-488 (green)[48]. A: Low-magnification overview of the peripheral cytoskeleton reveals actin-rich lamellipodia and associated filopodial extensions. Note the numerous GM1-positive lipid rafts interspersed throughout the extracted cytoplasm. The corresponding high-magnification CFEM analysis (a1-a3) of one of these lamellipodium-filopodial regions reveals the complex architectural nature of the leading edge, showing an intricate cytoskeleton-rich matrix and the close structural relationship with the lipid rafts (a1). Subsequent TEM analysis of exactly the same region not only allowed us to determine the exact size and shape of lipid rafts, but also provided an accurate idea of whether the rafts were located on top or beneath the lamellipodium: i.e. apical or basal (a2). The corresponding merged information (a3) clearly shows the additional value of applying different imaging techniques on the same cell: the electron-microscope data reveals small detergent-resistant membrane islands that could not be resolved by advanced confocal microscopy. Scale bar, 2 μm; For the electron tomographic analysis of a detergent-resistant membrane island and the surrounding cytoskeletal matrix (show in B and C), whole mounts of Caco-2 cells were prepared as outlined under A. Then we performed transmission electron tomography imaging at 1.5° incremental steps under dual-axis tilting (B) and then carried out subsequent segmenting (b1-b4) of the XYZ-tilting series. The single-image slices obtained *via* the IMOD tomography software show the sample at different heights from bottom to top (b1-b4) spanning a height of about 300 nm; C: An area of the entire tomogram was next selected (c) and a three-dimensional model generated of the membrane raft-cytoskeleton interface (c), showing the close interaction of fine cytoskeletal fibers at the rim of the detergent-resistant membrane.

cases, no fluorescent label could be detected at all, supporting earlier observations regarding the heterogeneous size and composition of lipid rafts[46,47]. The samples prepared for CFEM investigation were also readily used for subsequent TET[48], which generated a stack of virtual XYZ sections through the structure of interest (Figure 1B). When

combined with computer modeling, we obtained a three-dimensional view of the structure at a typical resolution of approximately 5-8 nm (Figure 1C). From these tomographic data, it became apparent that a web of fine cytoskeletal fibers, derived from the surrounding cytoskeletal matrix, accumulates at the circumference of rafts. These cytoskeleton-raft interactions probably indicate that membrane rafts require an intact cytoskeleton lattice for proper functioning of raft-associated subcellular processes[49].

Raft-mediated cell death

There has been an extraordinary increase in research activity aimed at understanding the mechanisms and processes that underlie cell death, which occurs in different forms. The best-studied cell death mechanisms are apoptosis and necrosis (for a recent review[50]). Whether by apoptosis or necrosis, cell death is initiated, triggered and regulated by a cascade of signaling pathways that involve different molecules. As we continue to unravel the pathways of cell death, novel findings emerge concerning the complex role that the cell membrane - and in particular rafts and their associated molecules - play in the tightly coordinated process of programmed cell death and cell survival[25]. A few reports unambiguously illustrate that rafts are key signal transducers when it comes to death of CRC cells. Remacle-Bonnet *et al*[51] showed that lipid rafts segregate pro- from anti-apoptotic insulin-like growth factor-I (IGF-I) receptor signaling in various human colon adenocarcinoma cell lines (HT29-D4, HRT-18, HCT-116, HCT-15, COLO-205, SW480, SW620, HCT-8/E11 and HCT-8/E11R1) when exposed to tumor necrosis factor-α (TNF-α). However, the pro-apoptotic effect of IGF-1 was not observed in all CRC cell lines tested, and SW480, SW620, HRT-18 and COLO-205 cells seem to be exceptions. The authors found that the paradoxical pro-apoptotic action of IGF-I is conveyed *via* the phosphoinositide 3-kinase (PI3K)/Akt pathway and that integrity of lipid rafts is necessary for proper anti-apoptotic cell signaling (Figure 2). In contrast, the activation of the Erk 1/2 and p38 MAPK pathways that transmit the IGF-I anti-apoptotic signaling is independent of lipid rafts.

These unexpected findings, obtained by incubating the different cell lines with the cholesterol-depleting agent methyl-β-cyclodextrin (Me-β-CD), showed the complicated functions that lipid rafts can display, depending on the molecules they are exposed to in the tumor microenvironment. Here, lipid rafts acted to precisely regulate whether CRC cells would survive or die. This might also partially explain the different and conflicting data reported in CRC cell death studies.

Another groundbreaking finding comes from a careful comparative *in vitro* and *in vivo* study in which sugar-cholestanol derivates provoked cell death in COLO-201, HT29 and Colon-26 cells, including Balb/c mice that contain Colon-26 tumors[38]. In this study, Hahismoto *et al* showed that chemically-synthesized sugar-cholestanols, with mono-, di- and tri-saccharides attached to cholestanol, are transported into the cell's interior *via* membrane microdomains; however, cholestanol without sugar moieties was not taken up. Biochemical analysis revealed that all N-acetyl-D-glucosamine-based sugar-cholestanols accumulate quite rapidly within the mitochondria of CRC cells, gradually increasing the release of cytochrome C from these organelles within the cytoplasm. Subsequent studies performed with time-pulse DNA ladder fragmentation assays and Western blotting demonstrated that cell death occurred *via* the caspase-9/caspase-3 apoptotic pathway (Figure 2). In their animal studies, the authors validated the potential anti-cancer effect of sugar-cholestanol derivates when administered intraperitoneally at different time intervals: Balb/c mice showed a significant reduction in tumor growth and had prolonged survival. This is one of the first CRC *in vivo* studies that unambiguously demonstrated the importance of membrane microdomains as a molecular target for cancer therapy (see also next section).

Other studies have shown that food-derived biochemical compounds can induce substantial cell death in CRC. In 2003, resveratrol - a polyphenol found in various food products - was reported to trigger apoptosis in SW480 human CRC cells[42]. By combining microscopy, cell sorting and protein blotting, the authors established the direct involvement of the caspase-8/caspase-3-mediated apoptotic cascade (Figure 2). Furthermore, resveratrol exposure induced a specific redistribution of the cell death receptor Fas (i.e. CD95) within membrane microdomains, and caused formation of the death-inducing signaling complex. Intriguingly, no interaction between the cell death receptor ligand (i.e. FasL) and Fas was required for the resveratrol-induced cell death. The authors, therefore, postulated that resveratrol, which is abundantly found in grape skin, holds strong potential as a chemoprotective and therapeutic agent for CRC and other malignant tumors. In another study, quercetin - a plant-derived flavonoid, plentiful in apples and red onions - was reported to induce apoptosis in HT-29 and SW-620 cells, although by a different apoptosis signaling pathway[40]. It was found that quercetin enhanced apoptosis caused by the TNF-related apoptosis-inducing ligand (TRIAL) through redistributing the death receptors (DR) DR4 and DR5 into membrane microdomains (Figure 2). The application of nystatin, a cholesterol-sequestering agent, prevented (1) quercetin-induced clustering of death receptors; and (2) sensitization to TRIAL-induced apoptosis in CRC cells. The involvement of the mitochondrial-dependent death pathway was demonstrated by the activation of related pro-apoptotic molecules and the subsequent release of cytochrome C to the cytosol. These data suggest that membrane microdomain localization of death receptors is probably required for optimal cytotoxicity of quercetin and/or TRIAL.

Cisplatinum or cisplatin is a well-known chemotherapeutic drug, widely used to treat various types of cancers. Rebillard *et al*[32] demonstrated that cisplatin-induced apoptosis in human CRC cells involves cell membrane fluidification *via* the inhibition of the Na^+/H^+ membrane exchanger-1. Inhibition leads to an overall intracellular acidification and the subsequent activation of acidic sphingomyelinase, which generate ceramides that finally affect membrane fluidity. The team also found that this

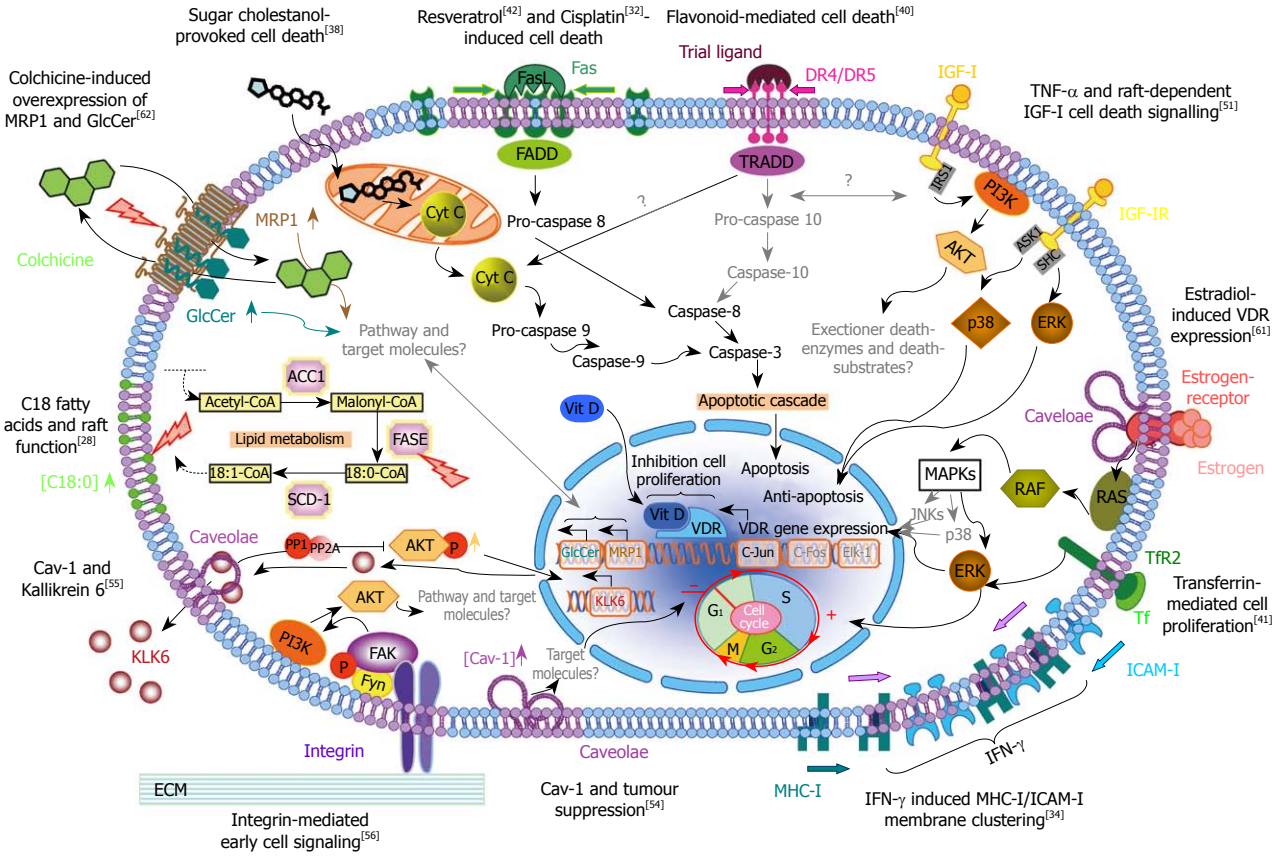

Figure 2 Scheme outlining the various membrane microdomain-mediated intracellular signaling pathways in colorectal cancer. This diagram summarizes what has been reported to date in the literature about the different intracellular signaling pathways that are mediated by lipid rafts and the implications of these paths for the colon cancer cells' state and fate. Briefly, the different observations of colorectal cancer (CRC) lipid rafts can be generally categorized under the following main topics of investigation: cell death-mediated mechanisms, caveolae in cancer cell growth and function, unique structure-function molecular associations, and intervention studies with bioactive compounds. Note that the text and connector arrows as shown in black are confirmed observations, whereas the gray denotes postulated signaling pathways and/or unknown molecular targets. The lipid bilayer of the cell membrane is depicted in light blue, membrane microdomains or lipid rafts in light purple, and the pear-shaped caveolae associated with these rafts in dark purple. For detailed descriptions of each of the individual cell signaling pathways, refer to the corresponding sections in this paper. The numbers in superscript refer directly to the original published papers. MRP: Multidrug-resistance protein; GlcCer: Glucosylceramide; FADD: Fas-associated protein with death domain; TRADD: Tumor necrosis factor receptor type 1-associated DEATH domain protein; PI3K: Phosphoinositide 3-kinase; AKT: Serine/threonine protein kinase; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen-activated protein kinase; IRS1: Insulin receptor substrate 1; ASK1: Apoptosis signal-regulating kinase 1; SHC: Src homology 2 domain; TNF-α: Tumor necrosis factor-α; IGF-Ⅰ: Insulin-like growth factor-Ⅰ; VDR: Vitamin D receptor; Vit D: Vitamin D; RAF: Proto-oncogene serine/threonine-protein kinase; RAS: RAt sarcoma; TfR2: The second transferrin receptor; Tf: Transferrin; JNKs: c-Jun N-terminal kinases; ICAM-Ⅰ: Intercellular adhesion molecule Ⅰ; IFN-γ: Interferon-γ; MHC-Ⅰ: Major histocompatibility complex Ⅰ; FAK: Focal adhesion kinase; ECM: Extracellular matrix; FASE: Fatty acid synthase; SCD-1: Stearoyl-coenzyme A desaturase 1; ACC1: Acetyl-CoA carboxylase; Cav: Caveolin.

rapid increase in membrane fluidity after cisplatin treatment was inhibited by membrane stabilizing agents, such as excess cholesterol or monosialoganglioside-1 treatment. Furthermore, these lipid-interfering compounds prevented the early aggregation of the Fas receptor and of membrane microdomains on the cell surface of HT-29 cells. As a result, significant inhibition of cisplatin-induced apoptosis was observed, without altering the intracellular drug uptake or the formation of cisplatin-DNA adducts. Hence, cisplatin-induced cell death in CRC cells seems to be mediated, in part, *via* the Fas-signaling pathway, the cell death receptors of which reside within lipid rafts (Figure 2). This concept was elegantly demonstrated through a variety of analytical tools, including GM1 labeling studies, in the course of lipid fluidity studies on isolated lipid rafts, and biochemical assays to detect caspase-3 activity and determine the extent of apoptosis.

Caveolin at the raft interface

There is widespread evidence that caveolin proteins associate structurally and functionally with membrane microdomains, interacting closely with numerous microdomain-associated molecules and thereby regulating cell signaling pathways that control cell function and cell fate[23]. Although all types of caveolins (Cav-1, Cav-2 and Cav-3) are structurally similar and associate with cholesterol and sphingolipids in parts of the cell membrane to form caveolae, Cav-1 and Cav-2 are most prominently upregulated and/or downregulated in oncogene-transformed cells[35]. However, a certain degree of variability in Cav-1 and Cav-2 expression seems to occur within various classes of cancer and across different types of cells or tissues. Furthermore, the amount of Cav-1 and Cav-2, together with the ultrastructural presence of caveolae, not only depends on the tumor model studied, but also on the stage and grade of

the cancer. This is particularly relevant for CRC in which the expression of Cav-1 mRNA is four to five times lower or two times lower, respectively, for Cav-2 mRNA in HT-29 or in COLO-205 CRC cell lines. These data were obtained by directly comparing the relative expression of caveolins in more than 55 commonly-used human cancer cell lines from different tissues (for an overview[35]). However, others have demonstrated, *via* classical immunocytochemical staining[52] and reverse transcriptase-polymerase chain reaction (RT-PCR)[53] in human colon tumor tissue, that Cav-1 was significantly enhanced compared to normal colon epithelium. In addition, caveolin-mediated raft signaling has been demonstrated to be pivotal in various experimental CRC models, especially in the strong anti-proliferative action of Cav-1. Bender *et al*[54] showed that Cav-1 possesses a strong tumor suppression activity in CRC cells, but the exact signaling mechanism responsible is not yet clear (Figure 2). Experiments involving immunoblotting, RT-PCR and microarrays disclosed that expression of Cav-1 in HT-29 and DLD-1 cells delayed or blocked tumor formation in nude mice. Likewise, Cav-1 levels were significantly reduced in colon tumors from human patients. Another interesting finding was that increased levels of Cav-1 were observed in multidrug-resistant HT-29 cells. The authors concluded from their studies that Cav-1 modulates a variety of signaling pathways, but that we still require a better understanding of target molecules affected by the expression of this all-round protein in malignant cells.

Henkhaus *et al*[55] demonstrated a direct link between Cav-1-mediated expression and secretion of kallikrein 6 (KLK6) in HCT116 human CRC cells. Sucrose-gradient subcellular fraction analysis revealed that Cav-1 and KLK6 co-localize to lipid rafts. Deactivation of Cav-1 - through interference in the Src-mediated phosphorylation pathway - decreases KLK6. In addition, immunoblotting, ELISA and RT-PCR studies revealed that Cav-1 controls KLK6 expression *via* the Src, Akt and phosphatases (i.e. PP1/PP2A) signaling pathway (Figure 2). Kallikreins are serine proteases, and the various subtypes of kallikreins are considered to hold promise as specific biomarkers for different cancers. Furthermore, once secreted by the cell, these serine proteinases have the ability to degrade the surrounding extracellular matrix (ECM). The authors postulated that KLK6-mediated degradation of the ECM enhances CRC cell invasiveness.

Raft-associated molecular expression

Besides the presence of cell death receptors and caveolar proteins within membrane microdomains, other key molecules have been shown to have close structure-function associations with rafts, thereby controlling a variety of other vital cellular processes in CRC, such as cell adhesion and motility, intracellular transport and cellular exchange, immune tolerance, and numerous hormone-mediated cellular responses[23]. For example, Baillat *et al*[56] demonstrated how focal adhesion kinase (FAK) and Src family protein tyrosine kinases (SFKs) work together in lipid rafts during the initial stage of CRC cell adhesion. It is well known that elevated expression and activity of SFKs in CRC often accompany disease progression. In cancer development, tumor cells acquire migration capability and effective homing ability in the body's host environment. Cell adhesion molecules, such as integrins that link components of the ECM with the cytoskeleton, are pivotal during this process. By applying a combination of cell transfection methods, protein-blotting assays and membrane raft ultracentrifugation to Me-β-CD-treated and/or cholesterol-treated SW480 cells, the team found that the formation of raft-associated FAK/Fyn complexes and the activation of Akt-1 *via* PI3K occur simultaneously during early contact with the ECM (Figure 2). Fyn is a tyrosine-specific phospho-transferase and member of the SFKs. Akt-1 is a serine threonine kinase that is considered to be an oncogene and is often activated in human cancers, thereby contributing to tumor progression and metastasis. The team concluded that, during the very early stage of cell adhesion, FAK is transiently co-located with Fyn thereby inducing raft-dependent Akt-1 signaling. This study also showed, for the first time, that FAK in membrane microdomains can act as a signaling intermediate to control various aspects of tumor cell behavior during cell adhesion.

Major histocompatibility complex I (MHC- I) and intercellular adhesion molecule I (ICAM- I) are crucially involved in the functioning of the immune system and are implicated in inflammatory bowel diseases and CRC. Cytokines used in therapy, such as interferon γ (IFN-γ), are known to modulate the expression of MHC- I and/or ICAM- I , so it is no surprise that these cell surface receptors are under investigation in an attempt to cure various gastrointestinal diseases. Bacsó *et al*[34] showed that exposing LS-174-T colon carcinoma cells to IFN-γ significantly increased the cell surface density of MHC- I and ICAM- I . Flow cytometric fluorescence energy-transfer measurements of immunolabeled LS-174-T cells and confocal microscopy of GM1-labeled LS-174-T cells revealed that both receptor types cluster in the nanometer range and that amounts of both substantially increase, within GM1-positive membrane microdomains, upon IFN-γ treatment (Figure 2). Moreover, the team found that the relative size of the lipid rafts increased, while the total cell size and membrane surface remained unchanged. Another interesting observation was that MHC- I and ICAM- I form sterically tight hetero-associates such that ICAM-1, with its long protrusion above the cell membrane, can readily bind to a cytotoxic lymphocyte (CTL) and simultaneously MHC- I can favorably present its peptide directly to the CTL. As both receptors are co-localized in lipid rafts, which are considered as pre-formed cell-signaling sites, all steric conditions are present for rapid trans-membrane signal transduction. These data imply that IFN-γ treatment can alter the surfaces of CRC cells to make them better target for CTLs.

Examination of resected tumor tissue has demonstrated that expression of the second transferrin receptor (TfR2) occurs in human colon carcinomas[41]. These authors also showed the presence of TfR2 in three different CRC cell lines (HT29, HCT116 and SKCO1) as assessed by immunolabeling, flow cytometry and Western blot analysis. This is intriguing given that TfR2 expression in

normal tissues is restricted to the liver, where it mediates cellular uptake of transferrin (Tf)-bound iron. The authors also found evidence that TfR2 expression induces a rapid and pronounced ERK1/ERK2 phosphorylation, indicating involvement of the Ras-dependent ERK1/ERK2 MAP-kinase signaling pathway (Figure 2). This pathway has a central role in controlling cell proliferation and is frequently activated in cancers, including CRC. It was concluded that TfR2 present within lipid rafts of CRC cells might contribute to the growth advantage of these cells.

Recently, Taïeb *et al*[57] demonstrated the presence of prominin 1 (CD133) within Caco-2 and HT-29-D4 cells. CD133 is a trans-membrane protein that has been shown, in different experimental tumor models, to make cancer cells transplantable, resistant against radiation therapy, and highly likely to initiate tumors. Researchers also believe CD133-ganglioside interactions are crucial for the recruitment and/or the phenotype of cancer stem cells. For this reason, there is obvious interest in understanding the molecular biology of $CD133^+$ cancer cells. With the aid of a novel anti-CD133 antibody, Taïeb *et al*[57] demonstrated that CD133-immunolabeling progressively decreased to undetectable levels in postconfluent CRC cell cultures, possibly through ganglioside-mediated epitope masking, because the staining was partially recovered after chemical disruption of lipid rafts. It is noteworthy that the N-terminal epitope of CD133 belongs to a ganglioside-binding domain and that blocking experiments with various gangliosides, including purified GM1, resulted in negative labeling. The authors proposed that synthetic soluble ganglioside analogues, which act as competitors and specifically affect $CD133^+$-mediated signaling pathways, deserve thorough evaluation in the development of new therapeutic approaches to CRC.

Bioactive compounds and raft function

Since the mid-1990s, hundreds of papers have examined the interference of drugs, pharmacological agents and other chemical or natural compounds with membrane microdomains[26]. Recent headway in animal disease models has provided knowledge as to how compounds affect membrane raft function, which might help to cure diseases such as ischemic heart impairment[58], keratitis[59] and colitis[60]. In colorectal studies, findings are limited so far to experimental *in vitro* models (see below). However, despite the limited data, the results all indicate that CRC membrane microdomains appear to be an important entryway for anti-cancer drugs, hormone(-like) molecules and dietary components. As reviewed above (see "Raft-Mediated Cell Death"), the bioactive compounds cisplatin[32], sugar cholestanol[38], flavoids[40] and resveratrol[42] have all been shown to possess a strong adverse cellular effect that is mediated *via* membrane raft signaling. Besides these classes of cell death-inducing compounds, other molecules have been described that control CRC cell function and fate.

For instance, in a study of the estrogen-induced vitamin D receptor (VDR) expression model, Gilad *et al*[61] elegantly illustrated how vitamin D (Vit D) controls CRC cell proliferation. By combining agents that interfere with lipid and intracellular signaling with subsequent protein immunoblotting studies, the authors unraveled that the estrogen 17β-estradiol (E2) binds to estrogen receptors (ERs) confined to lipid rafts or caveolae in HT29 cells, thereby activating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway *via* the protein kinases Ras/Raf. This affected transcriptional activity and finally resulted in upregulation of expression of the *VDR* gene. The MAPK/ERK pathway links intracellular responses to the binding of hormones and/or cell growth factors to cell membrane receptors (Figure 2). A direct functional association of ERs with lipid rafts was shown by using the cholesterol-binding agent Me-β-CD that blocked ERK phosphorylation concomitantly with VDR upregulation. E2 treatment did not affect proliferation of HT29 cells, while Vit D exposure significantly inhibited cell proliferation, and the combined treatment resulted in potentiation of Vit D activity. This anti-proliferative effect of Vit D, mediated *via* membrane microdomain receptor signaling, again illustrates the significance of lipid rafts in the regulation of tumor growth. It also emphasizes the importance of a well-balanced diet, including the daily intake of essential vitamins such as Vit D abundant in dairy products and fish oils, for controlling health.

Multidrug resistance (MRD) is a major challenge for drug designers and cancer cell biologists. When continuously exposed to chemotherapeutic compounds over long periods, cancer cells tend to acquire MDR by overexpressing proteins that belong to the superfamily of ATP-binding cassette (ABC) transporter proteins. Klappe *et al*[62] generated a new $HT29^{col}$ cell line that, during colchicine-induced acquisition of MDR, increases their glucosylceramide (GlcCer) content and upregulates multidrug-resistance protein I (MRP- I). The overexpression of sphingolipids, such as GlcCer, appears to be a rather general aspect of MDR cancers and has even been proposed as a candidate marker for MDR^+ malignant tumor cells. Furthermore, the tightly coordinated upregulation of MRP- I - a member of the ABC transporter proteins that displays strong drug-efflux properties - and GlcCer were both enriched in lipid rafts (Figure 2). The authors also demonstrated that GlcCer upregulation did not appear to be necessary for MRP- I function, given the absence of effects of inhibition of GlcCer biosynthesis on MRP- I -mediated drug efflux and cell survival. They, therefore, concluded that GlcCer appears to play a structural role in membrane microdomain organization instead and, as such, might help to accommodate the excess MRP- I expressed in membrane lipid islets in CRC cells.

The enzyme fatty acid synthase (FASE) synthesizes only saturated fatty acids and overexpression has been shown to be involved in numerous human malignancies, including CRC. Rakheja *et al*[28] demonstrated, through gas chromatography and mass spectrometry, a statistically significant increase in saturated C18:0 fatty acid (stearic acid) in colonic adenocarcinoma, compared to adjacent normal colonic mucosa tissue. None of the thirteen patients investigated had received any pre-operative chemotherapy and/or radiotherapy, thereby excluding artifactual read-

ings or false-positive measurements caused by treatment. Although the authors did not present any immunohistochemical data on normal or cancerous tissues, they postulated that the increase in the proportion of saturated fatty acids will most likely affect the functional properties of lipid rafts in CRC cells (Figure 2), and particularly might impair intracellular signaling mechanisms as discussed earlier (i.e. cell growth and cell death). In support of this proposal, it is known that the relative abundance of saturated fatty acids is a principle reason for the liquid-ordered state of lipid rafts and that the inhibition of FASE mainly affects the synthesis of raft-associated lipids. The authors concluded that dietary intervention to normalize the balance between saturated and unsaturated lipids within the cell membranes of colonic mucosa might be seen as a preventive or even therapeutic cure (see next section).

POTENTIAL DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS OF MEMBRANE MICRODOMAINS IN CRC

Many benign and malignant tumors synthesize and secrete compounds (i.e. tumor-associated proteins) that can be detected histopathologically on tissue sections or biochemically by chemical pathology analysis of blood or other bodily fluids. As the majority of these compounds are produced within tumors, they are said to be tumor-derived and so provide direct evidence of the tumor's existence (i.e. they are tumor-associated markers). It is highly probable that determination of raft composition or the detection of raft-associated markers, *via* proteomic and/or lipidomic approaches, could be extremely helpful in diagnosing malignancy in a patient with symptoms, preferably during the early stages of tumor formation[63,64]. The measured marker concentration or a combination of markers should directly correlate with the mass and/or activity of the tumor, and ideally might even help to fine-tune the formulation and dosages of anti-cancer drugs. Taking the currently available information on CRC rafts into account, the ratio of Cav-1/GM^+ and/or the presence of other molecules - such as TfR2, CD133 or KLK6 - have proven to be extremely useful candidate cancer markers that could aid in the diagnosis, staging and prognosis of CRC. However, despite initially high enthusiasm when the first experimental tumor marker-based assays appeared, only rarely does a marker exhibit sufficient specificity and sensitivity to be of any practical use in the clinic. While one single marker will not be sufficient to make a reliable diagnosis and prognosis, mass-spectrometry-based lipidomics[64] seems to hold great promise as a novel diagnostic approach to detect the unique "lipid fingerprint" of cancer cells. When combined with existing biomarker assays, lipidomics brings us one step closer to better monitoring and treating of cancer patients.

As it stands, we still have a long way to go when it comes to implementing our practical knowledge of lipid rafts to treat patients. Although the first animal studies showed much promise - as briefly mentioned before - in helping find a cure for diverse diseases, we still await the first full translational (pre-)clinical studies for curing diseases by targeting lipid rafts and their associated molecules. This, however, does not mean that raft-mediated therapy is entirely impossible or merely wishful thinking. It is just a reflection of the early days of membrane raft biology. When it comes to cancer and membrane raft-mediated therapeutic intervention, commonalities in proposed therapeutic approaches can be found that are independent of the cancer model studied. The majority of work targets trans-membrane proteins present in lipid rafts that control cell death *via* the programmed pathway (i.e. apoptosis; for a review[65]). However, this enthusiasm must be tempered by caution, because cancer cells have the ability to change the expression pattern of membrane-associated cell death receptors, depending on their cellular environment and/or progression stage. CRC cells have the ability, for example, to switch between the cell death receptor Fas and its corresponding ligand (i.e. FasL), depending on their microenvironment or the chemokines they meet[66,67]. A therapeutic intervention that uses smart drug complexes to target several different cell death molecules, including membrane raft-disrupting compounds, therefore is the approach most likely to result in successful outcomes.

Another interesting field concerns the modulation of membrane raft composition *via* dietary intake, or altering lipid synthesis *via* pharmacologic intervention within malignant cells[68]. Both are meant to change the cholesterol content within the lipid membranes in the hope of impairing the membrane raft signaling that controls tumor development and growth. Altered levels of membrane cholesterol and cholesterol-rich membranes have been shown to influence the aggressiveness and progression of cancers[69]. In CRC, this approach has a considerable chance of success because of the cancer's position within the digestive tract, which allows clinicians to directly expose the cancer cells to significant amounts of dietary or pharmacological compounds. In animal studies of various tumor models, dietary supplementation with long-chain polyunsaturated fatty acids combined with anti-cancer drugs significantly decreased tumor size and resulted in prolonged survival (for a review[70]). These fatty acids have been shown to be beneficial in various colon-related diseases, so an important role has been postulated for membrane microdomains in controlling tumor growth; this is currently a topic of active investigation[29,71]. Other examples of potential dietary therapies, as previously discussed, include the reduction of CRC tumor growth mediated by sugar-cholestanols[38], or the beneficial effects of polyphenols, found in grape skin and various other food products[42], or of quercetin, which is present in apples and red onions[40]. Whatever the ultimate therapeutic approach will be, it is most likely that a combination of drugs, combining classic anti-cancer pharmacological agents with anti-membrane raft compounds, has the best chance for success against the cancer cells' unfortunate ability to change their cell membrane composition to resist anti-cancer drugs. Indeed, it is not inconceivable that the same molecular drift will occur in membrane microdomains of cancer cells after continuous exposure to a certain class of drugs. The quest to identify

stable membrane raft molecular markers and/or target molecules within the different stages of tumor development and progression is the greatest challenge that faces cancer membrane biologists today.

CONCLUSION

Membrane microdomains or lipid rafts in CRC cells appear to be involved in all key cellular regulatory processes that control tumor development, growth, progression and regression. Signaling pathways for lipid metabolism, drug resistance, cell death, cell division and many other processes all seem to diverge from those peculiar lipid islands, thereby orchestrating the cancer cells' state and fate. This demonstrates once more the multifaceted nature of lipid rafts. In the near future, we foresee that manipulating the structural and functional integrity of lipid rafts with anticancer drugs might result in the direct inhibition of CRC cell adhesion, the arrest of cancer cell division, or might even totally eliminate cancer cells. One can only dream.

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REVIEW

State-of-the-art imaging techniques in endoscopic ultrasound

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Abstract

Endoscopic ultrasound (EUS) has recently evolved through technological improvement of equipment, with a major clinical impact in digestive and mediastinal diseases. State-of-the-art EUS equipment now includes real-time sono-elastography, which might be useful for a better characterization of lesions and increased accuracy of differential diagnosis (for e.g. lymph nodes or focal pancreatic lesions). Contrast-enhanced EUS imaging is also available, and is already being used for the differential diagnosis of focal pancreatic masses. The recent development of low mechanical index contrast harmonic EUS imaging offers hope for improved diagnosis, staging and monitoring of anti-angiogenic treatment. Tridimensional EUS (3D-EUS) techniques can be applied to enhance the spatial understanding of EUS anatomy, especially for improved staging of tumors, obtained through a better assessment of the relationship with major surrounding vessels. Despite the progress gained through all these imaging techniques, they cannot replace cytological or histological diagnosis. However, real-time optical histological diagnosis can be achieved through the use of single-fiber confocal laser endomicroscopy techniques placed under real-time EUS-guidance through a 22G needle. Last, but not least, EUS-assisted natural orifice transluminal endoscopic surgery (NOTES) procedures offer a whole new area of imaging applications, used either for combination of NOTES peritoneoscopy and intraperitoneal EUS, but also for access of retroperitoneal organs through posterior EUS guidance.

Key words: Endoscopic ultrasound; Real-time sono-elastography; Contrast-enhancement; Tridimensional (3D); Hybrid imaging; Endoscopic ultrasound-guided fine needle aspiration

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INTRODUCTION

Endoscopic ultrasound (EUS) has evolved in recent years into a technique with a major clinical impact in digestive and mediastinal diseases. Thus, EUS determines a change in the diagnosis in approximately a quarter of patients, as well as a change in management in half of the patients examined[1]. A major step in the development of the EUS imaging techniques (Table 1) was represented by the appearance of electronic linear EUS scopes, which allowed a significant improvement in image quality, as well as the

development of several EUS-guided or EUS-assisted procedures, which start with the real-time targeted placement of a fine-needle aspiration needle under direct imaging by ultrasound guidance[2].

REAL-TIME SONO-ELASTOGRAPHY

Technique

Real-time sono-elastography (RTSE) represents a technique which allows the calculation and visualization of tissue strain and hardness based on the average tissue strain in a selected region of interested[3]. The technique allows the real-time visualization of the calculated strain values (Figure 1A), displayed in a transparent layout over the gray-scale images, in a similar fashion with color Doppler imaging[4]. Several generations of software led to the improvement of image quality, reduced artifacts, but more important to the possibility of averaging through several cycles and calculation of semi-quantitative values of tissue strain inside a defined region of interest (for e.g. a lymph node or a focal pancreatic mass). By obtaining average hue histogram values inside a region of interest, the system displays the average strain inside a defined region of interest, as a semi-quantitative value that estimates tissue elasticity at that level.

Applications

Several applications were described for RTSE, as a technique that offers additional information as compared with gray-scale EUS images[5]. The technique allows the selection of the most probable lymph nodes to be malignant, as well as the identification of lymph nodes that are most probable to be benign[5-7]. This was suggested to be helpful for the selection and guidance of EUS-guided fine needle aspiration (FNA) for staging purposes in lung cancer or other digestive and mediastinal cancers (including esophageal, gastric or pancreatic cancer). EUS elastography was also reported to be useful for the differentiation of focal pancreatic masses, especially in pseudotumoral chronic pancreatitis and pancreatic cancer, in the presence of negative (false-negative) EUS-guided FNA and a strong suspicion of pancreatic cancer[8-11]. The results of initial studies were recently validated in two multicentre studies[12,13]. Both studies indicated similar values for sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy (92.6% *vs* 92.3%, 71.7% *vs* 80%, 76% *vs* 77.4%, 90.9% *vs* 93.3% and 87.4% *vs* 89.2%, respectively). It was thus suggested that the overall accuracy of 85-90% of EUS elastography might change current clinical decision making algorithms for the patients with focal pancreatic masses, especially in false-negative cases of EUS-FNA, when the suspicion of pancreatic cancer is still strong[12]. This warrants a more aggressive approach in negative EUS-FNA cases where EUS elastography suggests a hard mass, with the patients referred directly to surgery or to repeat EUS-guided FNA.

The method was also tested in initial feasibility studies in diffuse pancreatic diseases like early chronic pancreatitis or autoimmune pancreatitis[14,15].

Future usage of RTSE as a technique that simulates virtual palpation might include distant transmission of information and simulation of tele-palpation by using haptic devices and systems. This could lead to a better educational tool in order to simulate intra-operative palpation, and could also help provide guidance for remote surgical laparoscopic and robotic techniques.

CONTRAST-ENHANCEMENT

Technique

The development and subsequent approval of blood-pool contrast agents was a major step forward for the development of contrast specific ultrasound techniques[16]. Several contrast agents are clinically available, including Albunex, Levovist and Echovist (first generation), as well as SonoVue, Sonazoid and Optison (second generation), *etc.* All of them are quite safe, without severe complications or long-lasting side-effects. The usage of second-generation microbubble contrast agents further improved the diagnostic capabilities, through a strong increase in ultrasound backscatter and enhancement of echogenicity during the dynamic assessment of small volume and slow velocity blood flow. The advantage of second generation microbubble contrast agents is that they are able to pass through the lungs, thus remaining confined to the intravascular space for a longer time. Also, because of the low solubility they are more stable with favorable resonance at low acoustic pressures, hence longer specific imaging in real-time.

Initial applications used spectral (pulsed) Doppler, color or power Doppler imaging, with contrast agents used as vascular signal enhancers[17]. Contrast agents can thus rescue non-diagnostic Doppler examinations by increasing the intensity of weak flow signals to detectable levels. The appearance of contrast specific ultrasound modes further allowed the cancellation of tissue signals and utilization of the non-linear response of microbubbles (especially the second generation harmonic). The development of low-mechanical index techniques consequently led to a significant improvement consisting of visualization of the dynamic enhancement pattern in real-time. The main advantage is the absence of motion artifacts caused by cardiac or respiratory movements, including also flash and blooming (overpainting) artifacts.

Conventional imaging applications

One specific use of contrast-enhancement techniques in EUS was to detect low-velocity, low-volume flow of pancreatic tumors, with emphasis on the differential diagnosis between focal pancreatitis and pancreatic cancer[18-23]. An initial feasibility study in a pig model showed that the use of contrast agents is possible during EUS, leading to improved visualisation of the splanchnic vasculature[18]. Several studies further showed that contrast-enhanced power Doppler EUS can be successfully used for the differential diagnosis of chronic pseudotumoral pancreatitis and pancreatic cancer, with a sensitivity and specificity higher than 90%, in the presence of hypovascular malignant tumors[19-23]. An initial study that used Optison in combina-

Table 1 Comparative assessment of new endoscopic ultrasound imaging techniques

EUS technique	Advantage	Disadvantage	Cost	Invasiveness
Real-time sono-elastography	Improved diagnosis of focal pancreatic masses	Assessment in large RCTs needed	Average	Minimal
Contrast-enhanced EUS	Improved diagnosis and staging of focal pancreatic masses	Assessment in large RCTs needed	Average	Minimal
3D-EUS	Moderate improvement of staging in pancreatic area	Limited assessment in clinical applications	Average	Minimal
Optical diagnosis	Improvement of real-time diagnosis	Limited assessment in clinical applications	High	Average
EUS-NOTES	Improvement of therapeutic options	Limited assessment in clinical applications	High	High

EUS: Endoscopic ultrasound; RCTs: Randomized clinical trials; 3D-EUS: Tridimensional EUS; NOTES: Natural orifice transluminal endoscopic surgery.

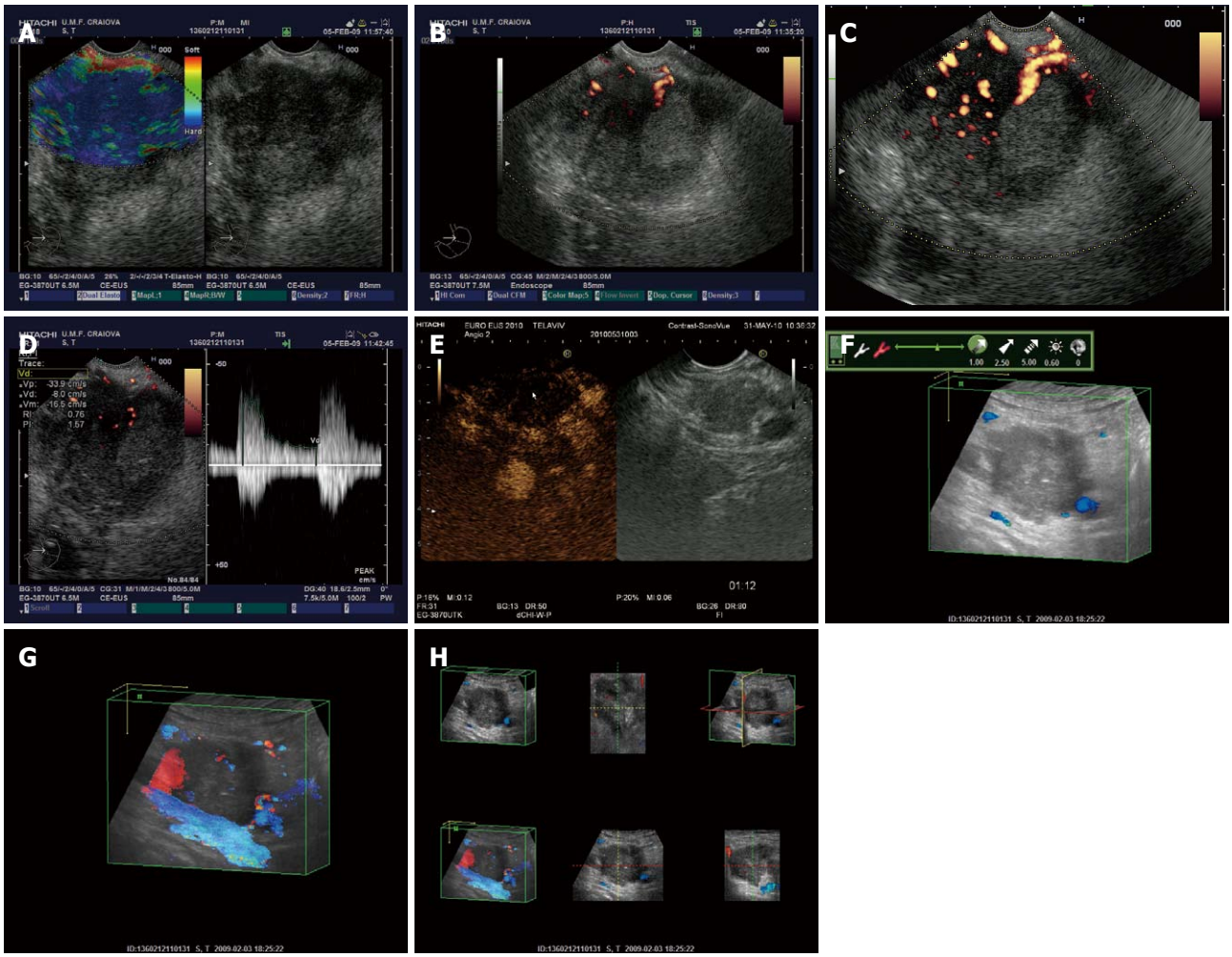

Figure 1 Pancreatic carcinoma at the level of pancreatic head depicted by different endoscopic ultrasound imaging techniques. A: Real-time elastography showing an in-homogenous hard mass; B: Power Doppler endoscopic ultrasound (EUS) without contrast-enhancement; C: Power Doppler EUS after contrast-enhancement with Sono-Vue, showing a hypovascular mass; D: Pulsed Doppler (triplex mode) after contrast-enhancement, with high resistivity and pulsatility indexes of intratumoral arteries; E: Contrast-enhanced low-mechanical index EUS harmonic imaging, showing a hypovascular appearance in the late (venous) phase; F: Tridimensional EUS showing an enhanced image in the opacity mode; G: Transparency mode obtained after contrast-enhancement with Sono-Vue; H: Multiview tridimensional (3D)-EUS display of the pancreatic tumor.

tion with power Doppler contrast-enhanced EUS showed a sensitivity, specificity, positive predictive value, negative predictive value and overall accuracy of 94%, 100%, 100%, 88% and 96%, respectively[19]. Another study used different criteria for benign and malignant lesions, after contrast-enhancement with Sono-Vue and power Doppler EUS examinations, in combination with pulsed (spectral) Doppler. Malignant lesions were defined by the presence of irregular arterial vessels over a short distance and no detectable venous vessels inside the lesion, while benign lesions included regular appearance of vessels over a distance of at least 20 mm after injection of SonoVue and detection of both arterial and venous vessels[20]. By using this methodology, the sensitivity and specificity for the detection of pancreatic cancer were 91.1% and 93.3%, with an overall accuracy of 91.9%. Two other studies con-

firmed these results, although they used Levovist, a first generation contrast agent, in combination with color or power Doppler[21,22]. By using power Doppler vascularity index values calculated by special software (Figure 1B-D), a recent study also depicted values of sensitivity and specificity of 90.5% and 90.1% for the characterization and differentiation of focal pancreatic masses after contrast-enhancement with Sono-Vue[23].

Harmonic imaging applications

Contrast harmonic imaging based on the second harmonic, in combination with microbubble specific software, allows an improved visualization of vascular and parenchymal phases, in a similar approach with computer tomography (CT) or magnetic resonance (MR) techniques. However, contrast harmonic imaging has several advantages and differences as compared with contrast-enhanced CT or MR, due to the different pharmacokinetics and containment inside the intra-vascular space of the ultrasound contrast agents[16]. The most important advantage is that contrast-enhancement patterns can be followed in real-time, with a very good temporal resolution (Figure 1E), while the administration can be easily repeated. Because the technology has recently become available for use during EUS examinations, a few studies have already assessed the value of contrast-enhanced harmonic EUS, based on second-generation contrast agents (mostly Sono-Vue)[24,25]. An initial feasibility study showed that harmonic EUS with low mechanical index can be used for the differential diagnosis of pancreatic cancer and chronic pancreatitis[24]. By using a mechanical index of 0.4 in conjunction with harmonic EUS with a low mechanical index, both real-time visualization of finely branching vessels of the pancreas, as well as intermittent parenchymal perfusion images could be obtained[25]. The method showed irregular "network like" structures inside the pancreatic carcinoma masses, with hypovascular heterogenous perfusion images in the intermittent mode. This contrasted with focal masses in chronic pancreatitis that were homogenous iso- or hypervascular, thus allowing a correct differential diagnosis.

The use of microbubble contrast agents has already been recommended for the monitoring of the response to anti-angiogenic treatment, because the conventional criteria [Response Evaluation Criteria in Solid Tumors (RECIST) and World Health Organization size criteria] do not show changes in the tumor parenchymal perfusion, hence they cannot predict response in the presence of tumor necrosis without volume changes[16]. Thus, a clear correlation has been proven between histological intratumoral microvessel density, vascular endothelial growth factor (VEGF) and microvessels visualized by contrast-enhanced ultrasound in pancreatic ductal carcinoma[26]. By using a similar approach, contrast-enhanced EUS might prove very useful for the real-time monitoring of the efficacy of antiangiogenic treatments. Contrast-enhancd harmonic EUS would certainly have the advantage of an improved resolution and decreased artifacts induced by bowel air and obesity.

Targeted imaging and targeted treatment

Microbubbles used as ultrasound contrast agents can be targeted *in vivo* to specific endothelial cell surface receptors[27]. Thus, different ligands can be conjugated to the outer surface of microbubbles and directed selectively towards endothelial cells. Microbubbles can be linked with monoclonal antibodies directed against VEGF receptor 2 (VEGFR2), thus allowing the binding to tumor-associated epithelium *in vivo*[28]. This allows *in vivo* quantification of VEGFR2 expression in tumor vessels, permitting both the selection of antiangiogenic drugs (e.g. bevacizumab which blocks specifically the VEGF-VEGFR2 pathway), as well as monitoring of treatment response. Multitarget quantification and visualization of targeted contrast-enhanced ultrasound microbubbles conjugated with either VEGFR2 and/or $\alpha(v)\beta(3)$ integrin were tested in initial experimental designs[29]. Targeted treatment during targeted contrast-enhanced ultrasound has been recently proven interesting due to the enhanced cellular uptake of drugs and genes in the presence of ultrasound and especially contrast-enhanced ultrasound, a process called sonoporation[30]. New microbubbles incorporating chemotherapeutic or gene vectors can be delivered at a cellular level through the formation of transient porosities in the cell membrane.

3D-EUS

Tridimensional EUS was recently reviewed in a separate paper[31]. The method enhances the spatial understanding of EUS anatomy, especially for the pancreatico-biliary area. The method can better depict the relationship with major surrounding vessels, consequently improving staging and resectability, mainly for pancreatic tumors (Figure 1F-H). Contrast-enhanced 3D-EUS can also be performed, allowing a better calculation of the vascular index that might offer important prognostic information, linked with the status before and after antiangiogenic treatment.

REAL-TIME OPTICAL DIAGNOSIS

Real-time optical pathological diagnosis might be achieved based on recent advances in single fiber-based optical techniques, the best example being confocal laser endomicroscopy[32]. Miniaturization of a confocal laser endomicroscopy miniprobe allowed the EUS-guided placement of a miniprobe through a 22G needle, inside different organs/lesions located in the vicinity of the digestive tract, e.g. pancreas, spleen, adrenal, liver, *etc.* The method has been shown to be feasible, yielding high quality confocal laser endoscopy images, equivalent of real-time histopathology images.

EUS-NATURAL ORIFICE TRANSLUMINAL ENDOSCOPIC SURGERY

The combination of EUS and natural orifice transluminal endoscopic surgery (NOTES) has already been described as a combination of NOTES peritoneoscopy and intraperitoneal EUS through transgastric and transcolonic ap-

proaches[33]. Thus, intraperitoneal EUS is considered safe and feasible, allowing adequate visualization of 4 sections of liver. Although objective landmarks for EUS were absent, intraperitoneal EUS could replace laparoscopic US, while NOTES peritoneoscopy can successfully replace laparoscopy. EUS-guided NOTES procedures were proven to be useful in a comparative sequential study which assessed mediastinoscopy/thoracoscopy, gastrojejunostomy and adrenalectomy[34]. EUS-guided access was useful mainly to obtain initial access or to identify structures in difficult areas, especially in the mediastinal or retroperitoneal regions. Furthermore, both an anterior and a posterior approach of the pancreas are possible through EUS-NOTES procedures, indicating a possible role for these combined techniques[35]. The aim was to improve pancreatic cancer staging of borderline cases and minimal invasive therapy of pancreatic diseases. Peritoneoscopy based on a EUS-assisted anterior transgastric approach, as well as EUS-guided posterior transgastric access to the pancreas, were both shown to be possible in this small non-survival animal study. Different therapeutic procedures like gastrojejunostomies and cholecysto-gastrostomies were also shown to be possible after initial EUS-assisted procedures. Future survival studies with randomized design should establish clearly the clinical role of these procedures.

CONCLUSION

EUS reached maturity as an imaging technique, as compared with the initial description in 1980. With a superior resolution as compared with cross-sectional imaging and with the addition of recent techniques like real-time sonoelastography, contrast-enhancement and 3D reconstructions, EUS seems likely to represent the technique of choice for early diagnosis, staging and stratification of prognosis. EUS-guided FNA or EUS-assisted procedures are also considered procedures of choice for the pathological confirmation of advanced cases, as well as for targeted treatment procedures. All of these procedures lead to a significant clinical impact of EUS, especially due to the improved clinical decision making algorithms, which nowadays incorporate routine EUS-guided or EUS-assisted procedures. The transition of these procedures to real-time optical diagnosis might offer additional value, allowing the immediate initiation of minimal invasive therapeutic procedures. Also, the appearance of combined EUS-NOTES procedures might enhance the safety and success of recent NOTES applications.

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REVIEW

Primary gastrointestinal lymphoma

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Abstract

Gastrointestinal tract is the most common extranodal site involved by lymphoma with the majority being non-Hodgkin type. Although lymphoma can involve any part of the gastrointestinal tract, the most frequent sites in order of its occurrence are the stomach followed by small intestine and ileocecal region. Gastrointestinal tract lymphoma is usually secondary to the widespread nodal diseases and primary gastrointestinal tract lymphoma is relatively rare. Gastrointestinal lymphomas are usually not clinically specific and indistinguishable from other benign and malignant conditions. Diffuse large B-cell lymphoma is the most common pathological type of gastrointestinal lymphoma in essentially all sites of the gastrointestinal tract, although recently the frequency of other forms has also increased in certain regions of the world. Although some radiological features such as bulky lymph nodes and maintenance of fat plane are more suggestive of lymphoma, they are not specific, thus mandating histopathological analysis for its definitive diagnosis. There has been a tremendous leap in the diagnosis, staging and management of gastrointestinal lymphoma in the last two decades attributed to a better insight into its etiology and molecular aspect as well as the knowledge about its critical signaling pathways.

INTRODUCTION

Gastrointestinal tract is the most common extranodal site involved by lymphoma accounting for 5%-20% of all cases[1]. Primary gastrointestinal lymphoma, however, is very rare, constituting only about 1%-4% of all gastrointestinal malignancies. Gastrointestinal lymphoma is usually secondary to the widespread nodal diseases. Although virtually lymphoma can arise from any region of the gastrointestinal tract, the most commonly involved sites in term of its occurrence are the stomach followed by small intestine and ileocecal region[2]. Histopathologically, almost 90% of the primary gastrointestinal lymphomas are of B cell lineage with very few T-cell lymphomas and Hodgkin lymphoma. Certain histological subtypes have been noted to have a relative predilection site as mucosa-associated lymphoid tissue (MALT) lymphoma in stomach, mantle cell lymphoma (MCL) in terminal ileum, jejunum and colon, as well as enteropathy-associated T-cell lymphoma (EATL) in jejunum, and follicular lymphoma (FL) in duodenum with a geographic variation in its distribution[3]. Multifocality, however, has been noticed particularly in MALT lymphoma and follicular lymphoma. Certain risk factors have been implicated in the pathogenesis of gastrointestinal lymphoma including *Helicobacter pylori* (*H. pylori*) infection, human immunodeficiency

virus (HIV), celiac disease, *Campylobacter jejuni* (*C. jejuni*), Epstein-Barr virus (EBV), hepatitis B virus (HBV), human T-cell lymphotropic virus-1 (HTLV-1), inflammatory bowel disease and immunosuppression[4,5]. Marker expression and translocations of common histological types of gastrointestinal lymphoma are depicted in Table 1.

Dawson's criteria are used for labeling primary gastrointestinal lymphoma, that include (1) absence of peripheral lymphadenopathy at the time of presentation; (2) lack of enlarged mediastinal lymph nodes; (3) normal total and differential white blood cell count; (4) predominance of bowel lesion at the time of laparotomy with only lymph nodes obviously affected in the immediate vicinity; and (5) no lymphomatous involvement of liver and spleen[6]. Ann Arbor staging with Musshoff modification is commonly employed to stage gastrointestinal lymphoma and the international prognostic index has been used to define the prognostic subgroups and Paris staging has increasingly gained its significance. Accurate diagnosis and staging of gastrointestinal lymphoma are detrimental for the stratification of treatment in this heterogeneous group of malignancies. The different procedures employed for the pre-treatment staging include endoscopic ultrasound (EUS), endoscopic biopsies, computed tomography (CT), magnetic resonance imaging (MRI), 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) or molecular markers[7,8]. Contrast-enhanced techniques and functional imaging such as perfusion CT can also help the monitoring, assessment, and prediction of response. New promising techniques such as hybrid PET-CT imaging and new PET tracers like 18F-fluoro-thymidine may significantly benefit the overall management of lymphomas[9].

In the following sections, the commonly involved sites of gastrointestinal lymphoma and its clinical, pathological and radiologic features are discussed with stress laid on the different histological subtypes based on the predilection. The characteristic features of gastrointestinal lymphoma in different regions of gastrointestinal tract are shown in Table 2.

CLINICAL/PATHOLOGICAL/IMAGING CHARACTERISTICS

Oropharyngeal lymphoma

The head and neck region is the second most common site for extra-nodal lymphoma accounting for 10%-15% of all cancers in this region. Approximately 2.5% of all malignant lymphomas originate from the oral and paraoral region, and the majority of them in the Waldeyer's ring include adenoids, palatine tonsils, base of tongue and oropharyngeal walls. Tonsil is the most frequently involved site (> 50%) of tumors, followed by nasopharynx and base of tongue[10]. Several factors are known to increase the risk of oropharyngeal lymphoma including EBV. The affected patients are usually at the age of over 50 years with a predilection of males. The most common clinical presentations of oropharyngeal lymphoma include airway obstruction, hearing pain, progressive enlarging painless local mass, dysphagia and foreign body sensation in the throat. Cervical lymphadenopathy is present in over 50% patients with tonsillar lymphoma[11].

More than 80%-90% of oropharyngeal lymphomas belong to the B-cell lineage of non-Hodgkin lymphoma (NHL)[12]. Diffuse large B-cell lymphoma (DLBCL) is the most common type of primary oral and paraoral NHL with a small percentage of thymic T-cell type. Histologically, DLBCL, composed of intermediate-large cells which may be noncleaved, cleaved and immunoblastic, shows B-cell lineage with expression of pan-B-cell antigens (CD19, CD20, CD22, CD79A, and PAX5/BSAP), and is less commonly positive for germinal centre cell markers (CD10 and BCL6) and negative for T-cell antigens. A small number of cases show a translocation between the *BCL-2* gene on chromosome 18 and the *IgH* gene on chromosome 14, t (14;18)[13]. Other lymphomas involving the Waldeyer's ring include 15% B-cell lymphomas in extranodal marginal zone of MALT, 8% peripheral T-cell lymphomas, 6% follicular lymphomas, and 3% MCLs. Hodgkin lymphoma (HL) involving the oropharynx is very rare accounting for about 1%-5% of all Hodgkin diseases. The majority of oropharyngeal HL are of lymphocyte predominant and nodular sclerosis type on histopathology with a common immunophenotype of Reed Sternberg cells positive for CD15, CD30 and negative for CD45, CD20, and EMA, which can rule out the diagnosis of NHL[14].

Radiologically, oropharyngeal lymphoma typically appears in barium studies as a lobular mass near the base of tongue in the palatine fossa with the overlying mucosa usually being nodular. The appearance of oropharyngeal lymphoma can be hard to differentiate from more common pharyngeal carcinomas. Because the signal intensity of lymphoma is similar to that of normal tissue, the MR signal characteristics cannot reliably show the early lymphomatous involvement at these sites. CT or PET with FDG and CT (PET/CT) has proved their usefulness both in diagnosis and staging of the disease and in assessment of its response to therapies[15]. Certain features that may favor the diagnosis of NHL on imaging are the short clinical history and a large homogeneous mass which displaces rather than invades local structures and large homogeneous non-necrotic cervical nodes[16].

Esophageal lymphoma

The esophagus is a rarely involved site, accounting for < 1% of all gastrointestinal lymphomas. Esophageal involvement usually results from metastasis from cervical or mediastinal lymph nodes or extension from gastric lymphoma. Primary esophageal lymphoma is extremely rare, with less than 30 cases reported in the literature[17-19]. The majority are the DLBCL type of NHL. Only few cases of MALT lymphoma, MCL, T-cell lymphoma and HL involving the esophagus have been reported[19-22]. The etiology of esophageal lymphoma is unknown and the role of EBV in its pathogenesis is controversial. It has been shown that esophageal lymphoma is most common in immunocompromised patients, with HIV infection as a probable risk factor[17]. The age of presentation is variable. The common symptoms

Table 1 Expression of common markers and translocations in histological subtypes of gastrointestinal lymphomas

Type	CD5	CD10	CD19	CD20	CD22	CD23	CD43	CD79a	CD3	CD7	CD4	CD8	CD30	CD15	CD45RO	Additional features
Diffuse large B-cell lymphoma	-(+)	-(+)	+	+	+	-	-	+	-	-	-	-	-(+)	-	-(+)	Bcl-6+(-), Bcl-2+(-), t (14;18), t (3;14), t (8;14)
MALT lymphoma	-	-	+	+	+	-	-(+)	+	-	-	-	-	-	-	-(+)	t (11;18), t (14;18), t (1;14), t (3;14)
Follicular lymphoma	-	+(-)	+	+	+	-(+)	-	+	-	-	-	-	-(+)	-	-	Bcl-2+, Bcl-6+, t (14;18)
Burkitt lymphoma	-	+	+	+	+	-(+)	+	+	-	-	-	-	-	-	-	C-myc, t (8;14)
Mantle cell lymphoma	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	Cyclin D1+, t (11;14)
Peripheral T-cell lymphoma-unspecified	+(-)	-(+)	-	-	-	-	+(-)	-	+(-)	-(+)	+(-)	-(+)	-(+)	-(+)	-(+)	-
Extranodal NK/T cell lymphoma	-(+)	-	-	-	-	-	+	-	-(+)	+(-)	-(+)	-	-(+)	-(+)	-	EBV+, gains of 2q, 15q, 17q, 22q, losses of 6q, 8p, 11q, 12q, 13q
EATL	-	-	-	-	-	-	+/-	-	+	+	-(+)	+(-)	+(-)	-	+(-)	TIA1+, gains of 1q, 5q, 7q, 9q, losses of 8p, 9p, 13q
Hodgkin disease	+(-)	-	-	-(+)	-	-	-	-(+)	+(-)	-(+)	-	-	+(-)	+(-)	-(+)	Variable

+: ≥ 90% positive; +(-): > 50% positive; -(+): < 50% positive; -: < 10% positive cases. MALT: Mucosa-associated lymphoid tissue; EATL: Enteropathy-associated T-cell lymphoma; EBV: Epstein-Barr virus.

of patients with esophageal lymphoma include dysphagia, odynophagia, weight loss, chest pain or present as a result of complications such as hemorrhage, obstruction or perforation with a tracheoesophageal fistula. Constitutional B symptoms (fever, night sweats) are not typically present.

Almost all cases of primary esophageal lymphoma are DLBCLs with positive surface markers of tumor cells on immunofluorescent staining for immunoglobulin G and κ light chain. MALT lymphoma of the esophagus, however, unlike that of stomach, is not associated with *H. pylori*. HL of the esophagus is extremely rare. Follicular lymphoma affecting the esophagus is a part of multifocal presentation in the gastrointestinal tract.

Radiological and endoscopic findings in esophageal lymphoma vary greatly and are nonspecific, which poses diagnostic challenges when it is differentiated from other benign and malignant lesions. Radiographic patterns of esophageal lymphoma, described in the literature[18-20], include stricture, ulcerated mass, multiple submucosal nodules, varicoid pattern, achalasia-like pattern, progressive aneurysmal dilatation, and tracheoesophageal fistula formation, and none of them is diagnostic. The morphological features seen at endoscopy are nodular, polypoidal, ulcerated or stenotic[21]. EUS has gained clinical acceptance for the assessment of lymphoma and preoperative staging, because it can accurately depict the structural abnormalities and depth of invasion of the lesions. EUS findings, however, are not pathognomonic, with presentation varied as anechoic, hypoechoic or even hyperechoic masses[22]. CT findings in esophageal lymphoma are nonspecific and not diagnostic, with features such as thickening of the wall mimicking other common tumors, such as esophageal carcinoma. CT, however, they are valuable for the evaluation of the extraluminal component of esophageal mass, its mediastinal extension, fistula formation, and status of lymph nodes, thus playing a role in staging disease, assisting in stratification of available treatment modalities, evaluating treatment responses, monitoring disease progression, and detecting relapses[23]. Recently, incorporation of PET/CT has emerged as an indispensable tool in staging the disease and following up the patients with extranodal involvement of Hodgkin's and non-Hodgkin's lymphoma, with an increased sensitivity and specificity. Diffuse large B-cell non-Hodgkin lymphoma of the esophagus is manifested as circumferential thickening of the wall, with diffuse increased FDG uptake. However, the intensity of FDG uptake in lymphoma is influenced by various intrinsic tumor factors such as histological features and grade, as well as various extrinsic factors. FDG PET/CT can also detect the indolent lesions that are undetectable on conventional cross-sectional imaging[24].

Gastric lymphoma

Stomach is the most commonly involved site (60%-75%) in gastrointestinal tract followed by small bowel, ileocecal region and rectum[25]. Gastric lymphoma accounts for 3%-5% of all malignant tumors of the stomach[26]. Although the incidence of gastric carcinoma has been reduced, the incidence of primary gastric lymphoma is increasing[27]. *H. pylori* play a role in the development of most MALT lymphomas. However, its exact mechanism has not been fully understood, although a chronic inflammation may enhance the probability of malignant transformation *via* B cell proliferation in response to *H. pylori* mediated by tumor-infiltrating T cells[28]. *H. pylori* may play a similar role in development of DLBCL and few studies have shown

Table 2 Characteristic features of gastrointestinal lymphomas

Region	Age (yr)	GIL (%)	Sex	Predilection site	Etiological/ risk factors	Presenting symptoms	Common pathological subtypes	Radiographic features
Oropharyngeal	> 50	-	M > F	Tonsil, nasopharynx[1], base of tongue	EBV	Dysphagia, dyspnea, painless mass, ulcer, oral/ hearing pain, B symptoms rare	DLBCL, EMZL/ MALT, PTCL, FL, MCL, ENKL, HD	Lobular mass, ulcers
Esophagus	Variable	< 1	-	Mid and lower third	EBV, HIV	Dysphagia, odynophagia, weight loss, epigastric/ chest pain, pneumonia, bleeding rare, B symptoms rare	DLBCL, MALT lymphoma, HD, MCL, T-cell lymphoma	Stricture, ulcerated mass, submucosal nodules, varicoid-like, achalasia-like, aneurysmal, fistula formation
Stomach	> 50	60-75	M > F	Antrum	*H. pylori* (MALT lymphoma), HTLV-1, HBV (DLBCL), EBV, HCV	Epigastric pain, dysphagia, nausea, vomiting, weight loss, abdominal mass, gastrointestinal bleeding, obstruction, perforation, B symptoms rare	DLBCL, MALT, PTCL	Ulcers, polypoid mass, thickened fold, mucosal nodularity, linitis plastica-like
Small intestine	Variable	20-30	Usually, M > F	Ileum, jejunum, duodenum, multiple sites	Celiac disease (EATL), *C. jejuni* (IPSID), EBV, HIV/ AIDS	Abdominal pain, nausea, vomiting, weight loss, GI bleeding, obstructive symptoms, intussusceptions, perforation, diarrhea (in IPSID), B symptoms rare	DLBLCL, MALT, EATL, MCL, Burkitt lymphoma, FL, IPSID, PTCL, ENKL	Polypoid mass, multiple nodules, infiltrative form, ulcer, excavation, fistulization, extraluminal mass, mucosal thickening, strictures
Colon/rectum	50-70	6-12	M > F	Caecum, ascending colon, rectum	Celiac disease (EATL), EBV, *H. pylori* (MALT lymphoma)	Abdominal pain, weight loss, abdominal mass, lower GI bleeding, obstruction, perforation	DLBCL, MALT, EATL, MCL, PTCL, Burkitt lymphoma	Polypoid mass, ulcers, mucosal nodularity, cavitary mass, mucosal thickening, strictures, aneurysmal

[1]Included here though usually not applicable. GIL: Gastrointestinal lymphoma; EBV: Epstein Barr virus; DLBCL: Diffuse large B cell lymphoma; EMZL: Extranodal marginal-zone lymphoma; PTCL: Peripheral T cell lymphoma; FL: Follicular lymphoma; EATL: Enteropathy-associated T-cell lymphoma; MCL: Mantle cell lymphoma; IPSID: Immunoproliferative small intestinal disease; HD: Hodgkin's disease; HTLV-1: Human T-cell lymphotropic virus-1; HCV: Hepatitis C virus; HBV: Hepatitis B virus; *H. pylori*: *Helicobacter pylori*; *C. jejuni*: *Campylobacter jejuni*; HIV: Human immunodeficiency virus; AIDS: Acquired immune deficiency syndrome; ENKL: Extranodal NK/T-cell lymphoma.

complete remission after eradication therapy alone[28]. It has been shown that individuals with positive HBsAg have an increased risk of developing NHL[29]. It was reported that HBV plays a role in the development of B-cell NHL[30]. In contrast, primary gastric lymphoma with a T-cell phenotype is relatively rare, accounting for only 7% of primary gastric lymphomas in HTLV-1 infected endemic areas and a relatively large number of such cases are secondary gastric involvement of adult T-cell leukemia. Primary gastric T-cell lymphoma without HTLV-1 infection is rare, and sporadic cases have been reported[31]. The age of most gastric lymphoma patients is over 50 years with a relative predilection in males. Clinical symptoms of gastric lymphoma are nonspecific and indistinguishable from other benign and malignant conditions. The most common complaints of gastric lymphoma patients are epigastric pain, weight loss, nausea and vomiting. Occasionally, an abdominal mass is palpable. Lymphadenopathy is rare and its patients often have no physical signs. Perforation, bleeding, or obstruction is very uncommon. Unlike nodal lymphoma, B constitutional symptom is not common.

Although all histological kinds of nodal lymphoma can arise from the stomach, the majority of them are of the B-cell origin, and MALT lymphoma and DLBCL account for over 90%. MALT lymphoma comprises up to 50% of all primary lymphomas involving the stomach. Histologically, the most significant finding is the presence of a variable number of lymphoepithelial lesions defined by evident invasion and partial destruction of mucosal glands by the tumor cells. MALT lymphoma shows the immunophenotype of B cells in the normal marginal zone of spleen, Peyer's patches and lymph nodes. The tumor B-cells can express the surface immunoglobulin and pan-B antigens (CD19, CD 20, and CD79a), the marginal zone-associated antigens (CD35 and CD21, and lack CD5, CD10, CD23) and cyclin D1. MALT lymphoma can be divided into *H. pylori* positive or negative based on the presence of *H. pylori*. *H. pylori* negative MALT lymphoma tends to have a higher positive rate for t (11;18) (q21;q21) translocation than *H. pylori* positive MALT lymphoma[32]. DLBCL, a heterogeneous group of tumors which are clinically, histologically, immunophenotypically, cytogenetically variable, can be divided into 3 subgroups, namely germinal-center B-cell-like, activated B-cell-like, and primary mediastinal DLBCL according to the gene expression patterns with each having a different prognostication. The most commonly seen translocations as mentioned earlier include t (14;18) (q32;q21) with BCL2-rearrange-

ment, t (3;14) (p27;q32) with BCL6-rearrangement and t (8;14) (q24;q32) with MYC rearrangement, respectively. Variability has been observed in CD45, CD5 and CD10 expression, with the CD10 expression in particular referred as a prognostic indicator[33].

Endoscopy cannot distinguish gastric lymphoma from the more common gastric carcinoma. The three main patterns that can be recognized at endoscopy include ulceration, diffuse infiltration, and polypoid mass, which are, however, not specific[34]. Endoscopy, however, is an indispensable tool for the initial diagnosis and follow-up of cases as well as for obtaining biopsy specimens. EUS can assess the extent of lesion and its invasion. Lesions are usually hypoechoic although few hyperechoic cases have been reported[34]. Infiltrative carcinoma tends to have a vertical growth in gastric wall, while lymphoma tends to show mainly a horizontal extension and more involvement of perigastric lymph nodes[35]. EUS is highly accurate in detecting the depth of lymphomatous infiltration and the presence of perigastric lymph nodes, thus providing additional information for treatment planning, and can differentiate lymphoma from carcinoma both in early stage and in advanced stage[36].

Radiographic patterns of gastric lymphoma observed in double-contrast UGI studies include ulcers, polypoid mass, thickened fold, mucosal nodularities or infiltrating lesions, which are not conclusive, thus posing a diagnostic challenge while differentiating from other malignant and benign lesions, hence requiring pathological confirmation. Preservation of gastric distensibility and pliability, despite the extensive infiltration with gastric fold thickening, is a finding more suggestive of lymphoma. Gastric wall thickening is much less severe in low-grade lymphoma than in high-grade lymphoma on CT images, and abdominal lymphadenopathy is less common in low-grade lymphoma. Preservation of the fat plane with no invasion of surrounding structures may be suggestive of lymphoma, although it is, however, not specific. Transpyloric spread and extension of lymphadenopathy below the renal hilum and the presence of bulky lymph nodes are more suggestive of lymphoma than carcinoma[37]. The patterns of gastric involvement observed can be segmental or diffuse infiltration, or localized polypoid. Tumor infiltration is usually homogeneous although areas of low attenuation may be present in larger tumors. Diffuse infiltration involving more than 50% of the stomach and segmental infiltration are the most common features of gastric NHL on CT images[38]. The MRI features include irregularly thickened mucosal folds, irregular submucosal infiltration, annular constricting lesion, exophytic tumor growth, mesenteric masses and mesenteric/retroperitoneal lymphadenopathy. The tumors are usually homogeneous and intermediate in signal intensity on T1-weighted images. Heterogeneously increased signal intensities are noted on T2-weighted images. The enhancement is usually mild-moderate after intravenous administration of gadolinium dimeglumine[39]. Application of 18F-FDG PET/CT in diagnosis of gastric lymphoma is challenging due to the physiologic FDG activity in the stomach and variability in the degree of uptake in various histologic subtypes. It was reported that aggressive gastric lymphoma has more intense uptake than low grade MALT lymphoma[40].

Small intestine lymphoma

Primary malignant tumors of the small intestine are very rare, accounting for less than 2% of all gastrointestinal malignancies. Lymphoma constitutes 15%-20% of all small intestine neoplasms and 20%-30% of all primary gastrointestinal lymphomas. Ileum is the most common site (60%-65%) involving small intestine lymphoma followed by jejunum (20%-25%), duodenum (6%-8%) and other sites (8%-9%)[41]. The age of presentation varies with the histological subtype of lymphoma. The clinical presentation of small intestinal lymphoma is non specific and the patients have symptoms, such as colicky abdominal pain, nausea, vomiting, weight loss and rarely acute obstructive symptoms, intussusceptions, perforation or diarrhea[42].

Primary small intestine lymphomas that are more heterogeneous than those in stomach include MALT lymphoma, DLBCL, EATL, MCL, follicular lymphoma and immunoproliferative lymphoma, and can be divided into immunoproliferative small intestinal disease (IPSID)[43]. IPSID, also known as alpha chain disease, is a MALT-associated lymphoma due to *C. jejuni* infection and characterized by "centrocyte like" mucosal infiltration with plasma cells that secrete monotypic and truncated immunoglobulin, a heavy chain lacking of an associated light chain. IPSID mainly affects older children and younger adults with a predominant involvement of proximal small intestine, the symptoms of its patients are diarrhea and abdominal pain[44]. MCL primarily affects individuals at the age of over 50 years, and involves terminal ileum and jejunum appearing as numerous polyps, hence called multiple lymphomatous polyposis[45]. The prototype MCL is positive for pan B-cell antigens, although few cases of CD5- MCL have been reported[44]. Cytogenetic analysis of MCL has shown the rearrangement of bcl-1 locus on chromosome11 due to t (11;14) (q13;q32) translocation, accompanying cyclin D1 antigen overexpression. Few cases of cyclin D1-negative MCL, however, have been reported with up-regulated cyclin D2 or D3[46]. Burkitt's lymphoma mainly affects children and is associated with EBV and HIV/AIDS[47]. T cell lymphoma of the small intestine accounts for approximately 10%-25% of all primary intestinal lymphomas primarily occurring as enteropathy-associated T cell lymphoma, and most of them are often complicated by Crohn's disease[48,49]. Although follicular lymphoma is very rare, it expresses SIg (frequently IgM) and pan B-cell antigens with CD10 and bcl-2 expressed in almost 90% of cases. It is negative for CD5 and cyclin D1 differentiating it from MCL. IgH/BCL2 rearrangement with t (14;18) (q32;q21) can be demonstrated by FISH or PCR analysis in the majority of cases[50]. Lymphocytic lymphoma (chronic lymphocytic leukemia) rarely arises primarily from the gastrointestinal tract.

Evaluation of the small intestinal lymphoma has been revolutionized since the introduction of capsule endoscopy (CE) and double-balloon technique of push-and-

pull enteroscopy which is capable of enabling biopsies as well as performing interventions, and limiting major surgical interventions. Small intestine lymphoma appears as a mass, polyp and ulcer on CE which cannot be distinguished from other lesions[51]. Radiologic findings of small intestinal lymphoma are not specific, thus posing a difficulty in distinguishing it from other benign and malignant lesions. The common features of small intestine lymphoma seen in barium studies and CT include polypoid form, multiple nodules, infiltrative form, endoexoenteric form with excavation and fistulization, and mesenteric invasive form with an extraluminal mass. The radiological findings usually do not correlate to its pathological subtypes. Certain features are, however, peculiarly noted. MCL, follicular lymphoma and MALT lymphoma rarely present with multiple polyps (multiple lymphomatous polyposis)[52]. Burkitt lymphoma usually presents as a bulky mass in the right lower quadrant. IPSID tends to affect proximally with a disseminated nodular pattern leading to mucosal fold thickening, irregularity and speculation. EATL, usually proximal or diffuse, shows nodules, ulcers or strictures[53]. PTCL preferentially involves the jejunum with an increased tendency to perforate[54].

Colorectal lymphoma

Colorectal lymphoma constitutes 6%-12% of all gastrointestinal lymphomas. Most colorectal lymphomas are secondary involvement of the wide spread diseases. Primary colorectal lymphoma is very rare, constituting only 0.2% of all malignant tumors arising from the colorectal region with caecum, ascending colon and rectum more often affected[55]. The disease predominantly affects males in the fifth-seventh decade of life with abdominal pain, loss of weight, palpable abdominal mass or lower gastrointestinal bleeding. Obstruction and perforation are relatively rare in patients with colorectal lymphoma[56].

Lymphoma of the colorectal region is mostly the B-cell lineage as other sites of the gastrointestinal tract. Primary colorectal lymphoma comprises low grade B-cell lymphoma arising from MALT, MCL and T-cell lymphoma besides large B cell lymphoma. The role of *H. pylori* in the pathogenesis of colorectal lymphoma has not been fully established[57]. Colorectal MALT-lymphoma is less common in colon and rectum than in small intestine. MCL in the colorectal region presents usually in the setting of diffuse systemic diseases. Peripheral T-cell lymphoma is rare in Western countries with an increasing frequency in many Asian countries, and is more aggressive in nature than other types with perforation as its common feature, and its prognosis is poor[58].

Endoscopically, lymphoma appears to be fungating, ulcerative, infiltrative, ulcerofungating, and ulceroinfiltrative types, with fungating and ulcerofungating types being more common[59]. The radiologic appearances of colorectal lymphoma are variable and significantly overlapped with other benign and malignant condition of the colorectal region. The imaging findings during double-contrast barium enema can be divided into focal and diffuse lesions. The observed focal lesions include polypoid mass, circumferential infiltration with smooth mucosal surface or extensive ulceration, cavitary mass, mucosal nodularity, and mucosal fold thickening. Diffuse lesions encompass diffuse ulcerative and nodular lesions. Peripheral T-cell lymphoma presents as a diffuse or focal segmental lesion with extensive mucosal ulceration similar to that observed in granulomatous conditions as Crohn's disease or tuberculosis. MALT lymphoma is manifested as multiple mucosal nodularity[60,61].

TREATMENT

The treatment strategy for gastrointestinal lymphoma is dependent on the age of patients, clinical scenario, histological subtype, extent and burden of the disease, and comorbidity, besides other factors. Surgery, chemotherapy, radiotherapy and radioimmunotherapy are the different modalities for its management and can be applied in different combinations. A detailed discussion on the treatment of all subtypes is beyond the scope of this article, thus the most common treatment modalities are highlighted in brief based on the region of presentation.

Oropharyngeal lymphoma

The definite management protocol for oropharyngeal lymphoma has not yet been established. Unlike the majority of other malignancies in this region, surgery does not play a primary role in the management of oropharyngeal lymphoma[62]. Combined chemotherapy and radiotherapy for localized oropharyngeal lymphoma is recommended in most studies[14]. Advanced oropharyngeal lymphoma is usually treated with aggressive chemotherapy with or without radiotherapy.

Esophageal lymphoma

Due to the rarity of esophageal lymphoma, no standardized approaches to its management have been formulated. Secondary lymphoma involving the esophagus can be treated with chemotherapy, while primary esophageal lymphoma can be managed with surgery, chemotherapy and radiotherapy or their combination. Treatment protocols vary depending on its histological subtypes and extent. Although surgery is the initial treatment modality, it has been recently reserved for cases with their diagnosis not possibly made at endoscopic biopsy or for those who warrant surgical intervention due to complications such as perforations. Esophageal lymphoma can be treated with local resection and chemotherapy with or without radiotherapy as its initial therapy. However, chemotherapy or radiotherapy alone can be also used as its initial therapy. The commonly employed chemotherapy regimen is CHOP in combination with Rituximab. It was reported that external beam radiation at the dose of 40 Gy can also be used[63].

Gastric lymphoma

Treatment strategies for gastric lymphoma have changed dramatically over the last two decades. However, they are still very controversial. The most widely recommended

strategy for the management of early stage *H. pylori* positive MALT type of gastric lymphoma is to eradicate *H. pylori* with antibiotics and proton pump inhibitors. Antibiotic therapy can achieve a long-term remission in 60%-100% patients with localized *H. pylori*-positive MALT lymphoma without t (11;18) chromosomal translocation. Histological assessment of treatment response, however, faces the problem of standardization, thus mandating serial follow-up. The GELA histologic evaluation system is commonly employed at certain centers. It has been shown that monoclonal B-cells still exist in almost half of the patients despite histological and endoscopic remission following antibiotic therapy[64].

No definite guidelines have been advocated for the treatment of advanced or *H. pylori* negative MALT-type of gastric lymphoma. Although surgery has been used as its initial treatment, recent studies showed that radiotherapy alone can achieve a complete remission with a 5-year disease free period[26]. Thus, "involved-field" irradiation at the total dose of 30 Gy for over 4 wk has become the treatment of choice for stages I and II MALT lymphoma without *H. pylori* or with persistent lymphoma following therapy. Surgery is, at present, reserved only for those with complications such as perforation, hemorrhage or obstruction that cannot be treated with other alternative therapies. Systemic therapy similar to that for indolent and advanced lymphoma must be taken into consideration in patients with their disease spread. Treatment options include chemotherapy and use of monoclonal antibodies. Diffuse large B-cell lymphoma of the stomach is treated with aggressive poly-chemotherapy, which is usually combined with Rituximab. Thus, gastric lymphoma should be treated with the front-line chemoimmunotherapy with 3-4 cycles of standard R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) followed by "involved-field" radiotherapy. A complete remission can be achieved in advanced gastric lymphoma patients after 6-8 cycles of R-CHOP as their nodal counterparts. Recent studies have demonstrated that anti-*H. pylori* therapy can achieve the remission of indolent lymphoma, *H. pylori* negative MALT lymphoma and DLBCL[26,65].

Intestinal lymphoma

The treatment outcome of intestinal lymphoma is relatively poorer than that of gastric lymphoma depending on their histologic subtypes. Lymphoma primarily located in the small intestine usually warrants laparotomy with the affected segment removed both for its diagnosis and for its treatment. Low-grade B-cell lymphoma of the small intestine (stage IE) only requires surgical resection. Although few studies have reported its benefit for localized intestinal lymphoma particularly that of the duodenum and rectum[66], radiotherapy in particular is not beneficial for intestinal lymphoma due to the multifocal involvement and its spread. No therapeutic guidelines are available for MALT lymphoma involving the small intestine with various modalities depending on the disease burden and other clinical parameters. Local intestinal lymphoma can be managed with surgical or endoscopic resection, while some cases of colonic MALT lymphoma can benefit from *H. pylori* therapy alone. Multi-agent chemotherapeutic strategy is warranted for advanced stage intestinal lymphoma with multifocal presentation of MALT lymphoma. A wait and watch policy for indolent FL at stage IE is advocated by some authors until they are symptomatic or show evidence of its progression due to a comparable relapse rate in treated patients and the progression of FL in untreated patients[50,66]. Symptomatic cases, or advanced disease of FL necessitates surgery, chemotherapy (CHOP) and/or irradiation intervention. Although Rituximab is beneficial for FL, its true value has not been well ascertained[66]. MCL treatment response and prognosis are poor with a short unmaintained remission after chemotherapy. Treatment is stratified based on the eligibility of patients for stem cell transplantation (SCT). Those who are eligible for grafting are previously induced with R-CHOP or R-HyperCVAD (Rituximab, cyclophosphamide, vincristine, doxorubicin and dexamethasone). Chemotherapy regimen, consisting of Rituximab alone or purine nucleoside analogs with Rituximab, can be applied to those ineligible for stem cell transplantation. The mammalian target of rapamycin inhibitors, antibodies, bendamustine or radioimmuno conjugates, can achieve a promising outcome in patients with relapse or refractory setting single-agent bortezomib, temsirolimus and ibritumomab tiuxetan[67,68]. IPSID in early stage responds to antibiotics such as tetracycline or combined metronidazole and ampicillin, with a remission occurring within 6-12 mo. IPSID at intermediate or advanced stage responds to anthracycline-based chemotherapy, with added antibiotics such as tetracycline. Surgery plays a limited role in the majority of cases due to diffuse involvement, although it may be requited for accurate diagnosis. It has been reported that radiotherapy as an adjuvant or palliative treatment is beneficial for some cases[66]. High grade lymphoma frequently presents with complications, thus mandating surgical intervention. No optimized therapeutic protocol is available for Burkitt lymphoma which usually requires an aggressive approach. High intensity chemotherapeutic agents for a short duration, such as cyclophosphamide, vincristine, doxorubicin, methotrexate and cytarabine, can significantly improve the treatment outcome. High dose chemoradiotherapy and hematopoietic SCT are beneficial for almost 50% of Burkitt lymphoma patients[69]. Radiotherapy is not beneficial for DLBCL involving the small intestine[70]. Systemic treatment with anthracycline-based chemotherapy followed by radiotherapy is proposed for wide spread advanced intestinal lymphoma which cannot be removed. Some studies have shown that post surgery chemotherapy is beneficial for some patients[68]. The overall response of non surgical patients with intestinal B cell lymphoma to chemotherapy is better than that of those with the intestinal T cell subtype[71]. No guidelines are available for the management of EATL although anthracyclin-based chemotherapy is a mainstay treatment modality for overt EATL with a poor response. In view of the poor performance and complications related to chemotherapy, such as perforation, multimodal approaches including curative or debulking surgery

Table 3 Paris staging system for primary gastrointestinal lymphomas

Stage	Gastrointestinal lymphomas
TX	Lymphoma extent not specified
T0	No evidence of lymphoma
T1	Lymphoma confined to the mucosa/submucosa
T1m	Lymphoma confined to mucosa
T1sm	Lymphoma confined to submucosa
T2	Lymphoma infiltrates muscularis propria or subserosa
T3	Lymphoma penetrates serosa (visceral peritoneum) without invasion of adjacent structures
T4	Lymphoma invades adjacent structures or organs
NX	Involvement of lymph nodes not assessed
N0	No evidence of lymph node involvement
N1	Involvement of regional lymph nodes
N2	Involvement of intra-abdominal lymph nodes beyond the regional area
N3	Spread to extra-abdominal lymph nodes
MX	Dissemination of lymphoma not assessed
M0	No evidence of extranodal dissemination
M1	Non-continuous involvement of separate site in gastrointestinal tract (e.g. stomach and rectum)
M2	Non-continuous involvement of other tissues (e.g. peritoneum, pleura) or organs (e.g. tonsils, parotid gland, ocular, adnexa, lung, liver, spleen, kidney, breast, *etc*.)
BX	Involvement of bone marrow not assessed
B0	No evidence of bone marrow involvement
B1	Lymphomatous infiltration of bone marrow
TNM	Clinical staging: status of tumor, node, metastasis, bone marrow
pTNMB	Histopathological staging: status of tumor, node metastasis, bone marrow
pN	The histological examination will ordinarily include six or more lymph nodes

are recommended to remove the gross EATL in all cases, if it is tolerable prior to chemotherapy[72]. It was reported that 66% of EATL patients undergoing surgical resection followed by combination chemotherapy and autologous stem cell transplantation can achieve a sustained complete response[73].

STAGING, PROGNOSTICATION AND RESTAGING

Staging of gastrointestinal lymphoma is a matter of debate due to various available staging systems. Although the modified Ann Arbor classification is feasible and relevant for prognosis, certain demerits in terms of disseminated and incurable infiltration of the gastrointestinal tract prompted development of the Paris staging system, which can differentiate distant lymphoma manifestations depending on the involved organ, and further subdivide lymph node involvement (Table 3)[74]. In general, comprehensive history taking and physical examination may reveal the possible etiologies of some specific lymphoma types and provide information for their further assessment and management. Minimal laboratory investigations performed include complete blood count, liver and renal function test, measurement of lactate dehydrogenase, blood glucose, serum uric acid, potassium, calcium, and phosphorus levels. Bone marrow aspirate with a biopsy is

Table 4 International prognostic index

Adverse risk factors
Age > 60 yr
⩾ 2 extranodal sites
Ann arbor stage Ⅲ-Ⅳ
Performance status ⩾ 2 (ECOG)
High lactate dehydrogenase
Risk
Low (*n* = 0-1)
Low-intermediate (*n* = 2)
High-intermediate (*n* = 3)
High (*n* = 4-5)

performed for involvement of lymphoma cells and monitoring of treatment response. Other investigations include serum protein electrophoresis and identification of paraprotein in certain types of lymphoma. Additional serological tests are often employed for etiological recognition in various types of lymphoma. CT scan of the chest, abdomen and pelvis is employed to stage gastrointestinal lymphomas with a marked sensitivity and specificity. Incorporation of FDG-PET has a significant advantage in staging of DLBCL, follicular lymphoma and MCL with a sensitivity of 80% and a specificity of 90%, although it has no added benefit for MALT lymphomas. EUS has gained momentum as an integral tool in the diagnosis, locoregional staging, and monitoring response of gastrointestinal lymphoma to treatment. EUS is superior to CT scan for the T- and N-staging by providing vivid details for any invasion to the mucosa, submucosa, muscularis propria or beyond serosa. The value of EUS and CT, however, is a matter of debate in the follow-up of patients as it is well established that histological remission precedes the normalization of wall changes in patients with lymphoma[74,75], thus precluding the necessity for endoscopic biopsy follow-up. Gastric MALT lymphoma, though indolent, often warrants a more meticulous staging procedure because it is usually multifocal, transforms to the DLBCL variant, and is difficult to diagnose due to normal endoscopic findings in the majority of cases as well as involvement of multiple organs. Endoscopic biopsies are therefore usually taken from multiple sites of the stomach and duodenum encompassing both normal and abnormal regions[75].

The international prognostic index (IPI) developed for DLBCL is, at present, the most valuable and widely used for the stratification of almost all subtypes of NHL (Table 4). However, the IPI does not hold the same predictive value for patients treated with immunochemotherapy. Moreover, the IPI is less useful for follicular lymphoma because a significant number of patients with a poor prognosis are not recognized, thus warranting development of follicular lymphoma IPI[76]. Reevaluation of patients who have completed the whole planned treatment is an integral part in the management of lymphoma patients. The most important prognostic factor for the management is the assessment of complete remission of the disease because salvage treatment with a high dose and autologous or allogenic bone marrow transplantation may be contem-

plated in those who fail to initial therapy. The different parameters are compared with the prior treatment values and evaluated. A possible necessity of histopathological assessment by follow-up biopsy may be required in certain atypical situations. Nuclear studies and PET in particular have been recommended for the evaluation of recurrence of various lymphomas[77].

FUTURE PERSPECTIVE

There has been a tremendous leap in the diagnosis, staging and management of gastrointestinal lymphoma in the last two decades. With a better insight into its etiology and molecular aspect, various critical signaling pathways provide an impetus with greater benefits. Identification of the cell surface antigens has led to the introduction of monoclonal antibodies like Rituximab and radioimmunotherapy that can result in a more targeted approach with a significant impact for the overall management of lymphoma. A deep understanding of the role of monoclonal antibodies in the pathogenesis of gastrointestinal lymphoma has led to development of the second and third generations of anti CD-20 antibodies (ofatumumab, veltuzumab, ocrelizumab), anti CD-22 antibodies such a Epratuzumab, anti CD-30 antibodies such as SGN-30, anti CD-40 antibody SGN-40, and anti vascular endothelial growth factor (VEGF) antibody bevacizumab[78]. Furthermore, addition of cytokines and other immune modulators has a boon resulting from a better understanding of the antibody activities at targeted tissues. Agents targeting the Bcl-2, Syk and the PI3K/AKT/mTOR pathways have emerged as a more biologically- focused management with further development in this field[79].

Another important aspect to be considered is the increasing sensitivity and specificity of imaging techniques like EUS and PET-CT in the diagnosis of lymphomas. An emerging field is the molecular imaging with a variety of new radiopharmaceutical agents that target the up-regulated specific receptors in cancer cells[80].

CONCLUSION

The epidemiology, clinical presentation, histopathologic subtypes, as well as radiological presentation of gastrointestinal lymphomas are highlighted in this review, with emphasis laid on the need for accurate diagnosis, staging, treatment of the disease with the promising novel techniques.

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ORIGINAL ARTICLE

Association of core promoter mutations of hepatitis B virus and viral load is different in HBeAg(+) and HBeAg(-) patients

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Abstract

AIM: To identify the prevalence of hepatitis B e antigen (HBeAg) and to assess the association of hepatitis B virus (HBV) core promoter mutations and viral load in Indonesian patients.

METHODS: Sixty-four patients with chronic hepatitis, 65 with liver cirrhosis and 50 with hepatocellular carcinoma were included in this study. HBeAg and hepatitis B e antibody (HBeAb) tests were performed using enzyme-linked immunosorbent assay and the mutations were analyzed by sequencing. Viral load was measured by real-time polymerase chain reaction.

RESULTS: Of 179 patients, 108 (60.3%) were HBeAg(-) and 86 (79.6%) of these HBeAg(-) patients had been seroconverted. The A1896 mutation was not found in HBeAg(+) patients, however, this mutation was detected in 70.7% of HBeAg(-) patients. This mutation was frequently found when HBeAg was not expressed (87.7%), compared to that found in HBeAg seroconverted patients (65.1%). The A1899 mutation was also more prevalent in HBeAg(-) than in HBeAg(+) patients (P = 0.004). The T1762/A1764 mutation was frequently found in both HBeAg(+) and HBeAg(-) patients, however, the prevalence of this mutation did not significantly differ among the two groups (P = 0.054). In HBeAg(+) patients, the T1762/A1764 mutation was correlated with lower HBV DNA (P < 0.001). The A1899 mutation did not correlate with HBV DNA (P = 0.609). In HBeAg(-) patients, the T1762/A1764 mutation alone was not correlated with HBV DNA (P = 0.095), however, the presence of either the T1762/A1764 or A1896 mutations was associated with increased HBV DNA (P < 0.001).

CONCLUSION: The percentage of HBeAg(-) patients is high in Indonesia, and most of the HBeAg(-) patients

had been seroconverted. The A1896 mutation was most likely the major cause of HBeAg loss. The T1762/A1764 mutation alone was associated with lower viral loads in HBeAg(+) patients, but not in HBeAg(-) patients.

Key words: Hepatitis B e antibody; Hepatitis B e antigen; Hepatitis B virus; Indonesia; Precore/core promoter mutations; Viral load

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INTRODUCTION

More than 2 billion people are infected with hepatitis B virus (HBV) and 350 million of them are chronic carriers of the virus[1]. Indonesia has a moderate to high endemicity of HBV infection, which is perhaps due to the lack of proper health facilities, poor economical status and less public awareness[2]. HBV infection is associated with a diverse clinical spectrum of liver damage ranging from asymptomatic carriers, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC)[3]. Patients with chronic hepatitis B are typically hepatitis B e antigen positive [HBeAg(+)] with detectable HBV DNA in serum. Generally, seroconversion from HBeAg to hepatitis B e antibody (HBeAb) positive correlates with reduced HBV replication in the liver and low infectivity during the natural course of infection[4,5]. In some patients, however, the immune pressure associated with seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop HBeAb, active HBV DNA replication continues with associated liver damage[6].

Most infected patients that are HBeAg(-) harbor HBV variants with mutations in the precore or core promoter region[7]. The predominant precore variation is a G-to-A change at A1896, which creates a premature stop codon and which abolishes the synthesis of HBeAg[8-10]. The most common core promoter mutations involve a two-nucleotide substitution at T1762 and A1764 (T1762/A1764 mutation)[7,11]. Several transfection studies showed that the T1762/A1764 mutation decreased the level of precore mRNA by 50% to 70% and led to reduced HBeAg synthesis[12-14]. A previous study has also demonstrated that HBeAg may be a target antigen on HBV-infected hepatocytes[15]. Failure to produce a target antigen may allow the infected cell to evade immune clearance.

The prevalence of HBeAg(-) patients is likely to vary across geographic areas. The total number of HBeAg(-) chronic hepatitis B patients is higher in the Mediterranean region and is estimated to be up to 33%. However, the prevalence of HBeAg(-) patients who achieved HBeAg seroclearance was higher in Asian patients (36%) than in Mediterranean patients (24%)[16]. Furthermore, HBeAg(-) patients have a higher rate of active liver disease in Asian patients, however, no data from Indonesia is available at present. In a recent study, we analyzed the genotype and core promoter mutations of HBV isolates in Indonesian carriers and patients[17]. The present study identifies the prevalence of HBeAg and assesses the association of HBV core promoter mutations and viral load in Indonesian patients.

MATERIALS AND METHODS

Patients

Serum samples were obtained from 179 HBV-associated liver disease patients, comprising 64 patients with chronic hepatitis B (CH), 65 patients with liver cirrhosis (LC), and 50 patients with HCC. Sera from CH, LC, and HCC patients were collected from Cipto Mangunkusumo Hospital, Gatot Soebroto Hospital, Klinik Hati "Professor Ali Sulaiman", Jakarta, Siloam Hospital Lippo Karawaci, Tangerang, Moewardi Hospital, Surakarta, Mataram General Hospital, Mataram, and M. Jamil Hospital, Padang, Indonesia, during the period of May 2006 to March 2010. All sera were persistently seropositive for HBsAg for at least 6 mo. The study was approved by the Institutional Ethics Committee and informed consent was obtained from each patient.

HBeAg and HBeAb tests

HBeAg and HBeAb from all plasma were tested using the MicroLISA™-HBeAg Test and MicroLISA™-HBeAb Test kits (Amgenix, San Jose, CA, USA), according to the manufacturer's instructions.

Analysis of HBV genotype and precore/core promoter mutations

HBV DNA was extracted from 200 μL serum using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and 80 μL eluted DNA was stored at -70℃ until use. HBV genotype was identified based on S gene sequence or genotype-specific polymerase chain reaction (PCR)[17,18]. Precore and core promoter mutations were analyzed by direct sequencing of the corresponding regions, as described previously[17]. However, for samples in which the precore sequence appeared inconsistent with the HBeAg test, amplification products were inserted into pBluescript Ⅱ SK(+), and at least ten independent clones of each were sequenced.

HBV viral load measurement

HBV DNA was first extracted from 200 μL serum with

Table 1 Demographics and characteristics of patients enrolled in this study

Characteristics	Total	CH	LC	HCC	*P*-value		
					CH *vs* LC	CH *vs* HCC	LC *vs* HCC
n (%)	179 (100.0)	64 (35.8)	65 (36.3)	50 (27.9)	-	-	-
Gender (male/female) (% male)	129/50 (72.1)	38/26 (59.4)	47/18 (72.3)	44/6 (88.0)	0.121	< 0.001	0.040
Age (yr, mean ± SD)	45.8 ± 12.3	39.7 ± 13.3	49.9 ± 10.8	48.4 ± 9.5	< 0.001	< 0.001	0.495
AFP [ng/mL, median (min-max)]	13.2 (0.1-3 295 000.0)	3.2 (0.1-5039.0)	11.8 (1.0-444 718.0)	704.8 (1.2-3 295 000.0)	< 0.001	< 0.001	< 0.001
AST [IU/L, median (min-max)]	66.0 (7.0-3618.0)	39.5 (7.0-481.0)	66.0 (15.0-297.0)	138.0 (9.0-3618.0)	< 0.001	< 0.001	< 0.001
ALT [IU/L, median (min-max)]	50.0 (1.0-860.0)	47 (6-748)	46 (9-216)	64 (1-860)	0.480	0.481	0.123
AST/ALT	1.28 (0.1-120.6)	0.9 (0.1-2.9)	1.4 (0.2-6.0)	2.1 (0.1-120.6)	< 0.001	< 0.001	< 0.001
Serum HBV DNA (log_{10} IU/mL, mean ± SD)	5.6 ± 2.0	6.1 ± 2.1	5.8 ± 1.4	4.6 ± 2.2	0.334	< 0.001	0.003
All HBeAg(+), (%)	71 (39.7)	36 (56.3)	19 (29.2)	16 (32.0)	0.002	0.010	0.749
All HBeAg(-), (%)	108 (60.3)	28 (43.8)	46 (70.8)	34 (68.0)			
HBeAg(+); HBeAb(-), (%)	71 (39.7)	36 (56.3)	19 (29.2)	16 (32.0)	0.002	0.010	0.749
HBeAg(-); HBeAb(+), (%)	86 (48.0)	22 (34.4)	40 (61.5)	24 (48.0)	0.002	0.141	0.039
HBeAg(-); HBeAb(-), (%)	22 (12.3)	6 (9.4)	6 (9.2)	10 (20.0)	0.978	0.105	0.098
Genotype							
B, *n* (%)	132 (73.7)	47 (73.4)	48 (73.8)	37 (74.0)	0.958	0.946	0.985
C, *n* (%)	47 (26.3)	17 (26.6)	17 (26.2)	13 (26.0)			

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody.

the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of internal control (1 μL/10 μL sample) from ARTUS HBV (Qiagen, Hilden, Germany). From 50 μL of eluted DNA, 20 μL was then quantified by ARTUS real-time PCR assay according to the manufacturer's instructions (Qiagen, Hilden, Germany). The range of HBV DNA detection was 10^1 to 10^5 IU/mL.

Statistical analysis

All statistical analyses were performed using SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA). Significance differentiations for continuous variables were analyzed using *t*-test analysis. The categorical variables were analyzed using the Fisher's exact test and χ^2 test. *P*-values of < 0.05 were considered significant.

RESULTS

Characteristics of patients

Patients' characteristics are summarized in Table 1. Of the 179 patients, 129 (72.1%) were male and the male/female ratio was significantly increased from CH to HCC and from LC to HCC, but not from CH to LC. The mean age of all patients was 45.8 ± 12.3 years, and significantly increased from CH to LC and to HCC, but not from LC to HCC. The median level of AST and ALT were 66.0 and 50.0 IU/mL, respectively. The level of AST, but not ALT, was significantly increased according to the severity of liver disease. The mean level of HBV DNA was 5.6 ± 2.0 log_{10} IU/mL, and was significantly lower in HCC (4.6 ± 2.2) than in LC (5.8 ± 1.4) and CH (6.1 ± 2.1). Seventy-one (39.7%) and 108 (60.3%) of patients were HBeAg(+) and HBeAg(-), respectively. The percentage of HBeAg(+) samples was significantly higher in CH (56.3%) than in LC (29.2%) or HCC (32.0). Eighty-six of 179 patients (48.0%) were HBeAg seroconverted, and the percentage of HBeAg seroconversion was higher in LC (61.5%) than HCC (48.0%) and CH (34.4%). There was no difference in HBV genotype prevalence between samples of different clinical diagnosis.

Comparison between HBeAg(+) and HBeAg(-)

Table 2 demonstrates the comparison between HBeAg(+) and HBeAg(-) groups. The mean age of patients was significantly higher in the HBeAg(-) group (49.2 ± 11.7) than in the HBeAg(+) group (40.7 ± 11.5) ($P < 0.001$), indicating a longer period of disease in the HBeAg(-) group. No significant difference in the male/female ratio between the two groups was observed ($P = 0.106$). Surprisingly, there was also no significant difference in ALT levels between the two groups ($P = 0.535$). As shown in Table 3, 79.6% (86/108) of HBeAg(-) patients were HBeAg seroconverted [HBeAb(+)]. There was no significant difference in ALT levels in the samples prior to HBeAg seroconversion [i.e. HBeAg(+), HBeAb(-)] *vs* after HBeAg seroconversion [i.e. HBeAg(-), HBeAb(+)] ($P = 0.200$). However, ALT levels in the HBeAg seroconverted group were significantly higher than those in the group that did not express HBeAg (60.0 IU/L *vs* 43.0 IU/L, $P = 0.023$). The AST/ALT ratio was significantly higher in HBeAg(-) than in HBeAg(+) patients (1.4 *vs* 1.1, $P = 0.022$), suggesting the presence of other factors involved in HBeAg(-) patients. HBV DNA was significantly higher in the HBeAg(+) (6.5 ± 1.8 log_{10} IU/mL) group compared to the HBeAg(-) (5.0 ± 1.9 log_{10} IU/mL) group ($P < 0.001$) (Table 2). The percentage of samples with HBV DNA load ≥ 20 000 IU/mL was much higher in HBeAg(+) patients (91.5%) than in HBeAg(-) patients (68.5%). There was no significant difference in levels of HBV DNA between the HBeAg sero-

Table 2 Comparison of hepatitis B e antigen (+) and hepatitis B e antigen (-) patients

Characteristics	Total	HBeAg(+)	HBeAg(-)	*P*-value
n (%)	179 (100.0)	71 (39.7)	108 (60.3)	-
Gender (male/female) (% male)	129/50 (72.1)	47/24 (66.2)	82/26 (75.9)	0.106
Age (yr, mean ± SD)	45.8 ± 12.3	40.7 ± 11.5	49.2 ± 11.7	< 0.001
AFP [ng/mL, median (min-max)]	13.2 (0.1-3 295 000.0)	10.9 (0.2-230472.0)	19.9 (0.1-3 295 000.0)	0.008
AST [IU/L, median (min-max)]	66.0 (7.0-3618.0)	55.0 (12.0-635.0)	71.0 (7.0-3618.0)	0.028
ALT [IU/L, median (min-max)]	50.0 (1.0-860.0)	47.0 (1.0-748.0)	52.0 (6.0-860.0)	0.535
AST/ALT	1.3 (0.1-120.6)	1.1 (0.1-30.8)	1.4 (1.4-120.6)	0.022
Serum HBV DNA (log_{10} IU/mL, mean ± SD)	5.6 ± 2.0	6.5 ± 1.8	5.0 ± 1.9	< 0.001
< 20 000 IU/mL, *n* (%)	40 (22.3)	6 (8.5)	34 (31.5)	< 0.001
≥ 20 000 IU/mL, *n* (%)	139 (77.7)	65 (91.5)	74 (68.5)	
Clinical status				
CH, *n* (%)	64 (35.8)	36 (50.7)	28 (25.9)	0.003
LC, *n* (%)	65 (36.3)	19 (26.8)	46 (42.6)	
HCC, *n* (%)	50 (27.9)	16 (22.5)	34 (31.5)	
Genotype				
B, *n* (%)	132 (73.7)	50 (70.4)	82 (75.9)	0.259
C, *n* (%)	47 (26.3)	21 (29.6)	26 (24.1)	

CH: Chronic hepatitis; LC: Liver cirrhosis; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B e antigen.

Table 3 Comparison of patients with hepatitis B e antigen (+), before and after hepatitis B e antigen seroconversion

Characteristics	Group 1	Group 2	Group 3	*P*-value		
	HBeAg(+), HBeAb(-)	HBeAg(-), HBeAb(+)	HBeAg(-), HBeAb(-)	1 *vs* 2	1 *vs* 3	2 *vs* 3
n (%)	71 (39.7)	86 (48.0)	22 (12.3)	-	-	-
Gender (male/female) (% male)	47/24 (66.2)	71/15 (82.6)	11/11 (50.0)	0.018	0.171	0.001
Age (yr, mean ± SD)	40.7 ± 11.5	49.8 ± 11.9	46.9 ± 10.6	< 0.001	0.028	0.296
AFP [ng/mL, median (min-max)]	10.9 (0.2-230472.0)	17.1 (0.1-3295000.0)	40.8 (0.4-514412.0)	0.016	0.048	0.731
AST [IU/L, median (min-max)]	55.0 (12.0-635.0)	68.0 (7.0-3618.0)	76.5 (9.0-580.0)	0.017	0.536	0.448
ALT [IU/L, median (min-max)]	47.0 (1.0-748.0)	60.0 (9.0-860.0)	43.0 (6.0-154.0)	0.200	0.173	0.023
AST/ALT	1.1 (0.1-30.8)	1.3 (0.1-120.6)	1.7 (0.5-6.0)	0.091	0.007	0.052
Serum HBV DNA (log_{10} IU/mL, mean ± SD)	6.5 ± 1.8	5.1 ± 1.8	4.5 ± 2.4	< 0.001	< 0.001	0.146
< 20 000 IU/mL, *n* (%)	6 (8.5)	25 (29.1)	9 (40.9)	0.001	< 0.001	0.286
≥ 20 000 IU/mL, *n* (%)	65 (91.5)	61 (70.9)	13 (59.1)			
Clinical status						
CH, *n* (%)	36 (50.7)	22 (25.6)	6 (27.3)	0.001	0.054	0.871
LC, *n* (%)	19 (26.8)	40 (46.5)	6 (27.3)	0.011	0.963	0.104
HCC, *n* (%)	16 (22.5)	24 (27.9)	10 (45.4)	0.442	0.036	0.114
Genotype						
B, *n* (%)	50 (70.4)	66 (76.7)	16 (72.7)	0.369	0.835	0.694
C, *n* (%)	21 (29.6)	20 (23.3)	6 (27.3)			

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; AFP: α-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBeAb: Hepatitis B e antibody.

converted and non-expressing groups ($P = 0.146$) (Table 3), suggesting that HBeAg expression did not affect HBV replication. The percentage of HBeAg(+) samples was high in CH (50.7%) and was less in LC (26.8%) and HCC (22.5%).

Precore/core promoter mutations and HBeAg status

The prevalence of mutations in the precore and core promoter regions was compared between HBeAg(+) and HBeAg(-) patients (Table 4). As expected, the precore A1896 mutation, which is associated with HBeAg expression, was absent in HBeAg(+) patients, but was found in 70.7% of HBeAg(-) patients. Interestingly, the A1896 mutation was frequently found not only in HBeAg non-expressing patients (87.7%), but also in HBeAg seroconverted patients (65.1%). The A1899 precore mutation was found to be more common in HBeAg(-) than in HBeAg(+) patients (34.5% *vs* 10.4%, $P = 0.004$), but there was no significant difference between the HBeAg seroconverted group and the HBeAg non-expressing group (37.2% *vs* 26.7%, $P = 0.116$), suggesting that this mutation contributes to the expression of HBeAg. On the other hand, the T1762/A1764 core promoter mutation was found both in HBeAg(+) (40.8%) and HBeAg(-) (55.6%) patients, and the prevalence of this mutation was not significantly different between the two groups ($P = 0.054$).

Table 4 Precore and core promoter mutations in patients with hepatitis B e antigen (+), before and after hepatitis B e antigen seroconversion *n* (%)

	All	Group 1	Group 2	Group 3	Group 2+3	*P*-value			
		HBeAg(+), HBeAb(-)	HBeAg(-), HBeAb(+)	HBeAg(-), HBeAb(-)	HBeAg(-)	1 *vs* 2	1 *vs* 3	2 *vs* 3	1 *vs* (2+3)
T1762/A1764[1]									
Absent	90 (50.3)	42 (59.2)	38 (44.2)	10 (45.5)	48 (44.4)	0.062	0.258	0.915	0.054
Present	89 (49.7)	29 (40.8)	48 (55.8)	12 (54.5)	60 (55.6)				
A1896[2]									
Absent	65 (61.3)	48 (100.0)[3]	15 (34.9)	2 (13.3)	17 (29.3)	< 0.001	< 0.001	0.114	< 0.001
Present	41 (38.7)	0 (0.0)	28 (65.1)	13 (87.7)	41 (70.7)				
A1899[2]									
Absent	81 (76.4)	43 (89.6)	27 (62.8)	11 (73.3)	38 (65.5)	0.003	0.034	0.116	0.004
Present	25 (23.6)	5 (10.4)	16 (37.2)	4 (26.7)	20 (34.5)				
T1762/A1764 and A1896[2]									
Absent	88 (83.0)	48 (100.0)	31 (72.1)	9 (60.0)	40 (68.9)	< 0.001	< 0.001	0.383	< 0.001
Present	18 (17.0)	0 (0.0)	12 (27.9)	6 (40.0)	18 (31.0)				
T1762/A1764 or A1896[2]									
Absent	33 (31.1)	31 (64.6)	2 (4.7)	0 (0.0)	2 (1.9)[4]	< 0.001	< 0.001	0.395	< 0.001
Present	73 (68.9)	17 (35.4)	41 (95.3)	15 (100.0)	56 (98.1)				

[1]Total samples *n* = 179; Hepatitis B e antigen (HBeAg) (+) and hepatitis B e antibody (HBeAb) (-) *n* = 71; HBeAg(-) and HBeAb(+), *n* = 86; HBeAg(-) and HBeAb(-), *n* = 22; [2]Total samples *n* = 106; HBeAg(+) and HBeAb(-) *n* = 48; HBeAg(-) and HBeAb(+), *n* = 43; HBeAg(-) and HBeAb(-), *n* = 15; [3]Three samples had a mixed population of precore stop codon mutation (A1896) and its wild type (G1896); [4]Two samples with a mixed population of basal core promoter mutation (T1762/A1764) and its wild type (A1762/G1764).

However, the presence of either the T1762/A1764 mutation or the A1896 mutation was very high in the HBeAg(-) group (98.1%), [including patients that had seroconverted (HBeAb(+)] (95.3%) and HBeAg non-expressing patients (100.0%), compared to the HBeAg(+) group (35.4% of which had the mutations), indicating these mutations are associated with HBeAg status.

We further analyzed the effect of precore and core promoter mutations on HBeAg expression and viral replication in twenty two HBeAg non-expressed samples. Enough DNA was recovered so that the T1762/A1764 core promoter mutation could be analyzed in all samples, however, the A1896 and A1899 precore mutations could only be analyzed in fifteen samples (Table 5). Either core promoter (T1762/A1764) or precore (A1896 or A1899) mutations were found in all samples, with the exception of three samples from which we were unable to obtain the DNA sequence. Almost all samples demonstrated relatively high HBV DNA, although in some samples the HBV DNA was low, but still detectable. Taken together, these results suggest that precore or core promoter mutations were associated with reduced HBeAg expression, but did not affect HBV replication in groups that did not express HBeAg.

Table 5 Precore and core promoter mutations in samples with hepatitis B e antigen (-) and hepatitis B e antibody (-)

No.	Sample ID	T1762/A1764	A1896	A1899	Serum HBV DNA (log_{10} IU/mL)
1	07.10.068	Yes	No	No	4.59
2	08.70.091	Yes	No	No	4.87
3	08.100.038	Yes	Yes	No	6.43
4	07.10.121	Yes	Yes	No	7.52
5	08.10.002	Yes	Yes	No	7.18
6	09.41.591	Yes	Yes	Yes	6.53
7	09.40.037	Yes	Yes	Yes	5.62
8	10.80.004	Yes	Yes	No	2.78
9	06.10.062	No	Yes	No	3.32
10	08.10.086	No	Yes	No	4.94
11	09.40.033	No	Yes	No	7.28
12	09.80.040	No	Yes	No	4.05
13	09.41.806	No	Yes	Yes	7.42
14	07.10.070	No	Yes	Yes	4.72
15	09.80.037	No	Yes	Yes	6.78
16	08.10.016	Yes	NA	NA	5.21
17	08.10.020	Yes	NA	NA	-0.37
18	08.10.039	Yes	NA	NA	3.10
19	P.X00.34	Yes	NA	NA	-0.54
20	07.10.117	No	NA	NA	1.73
21	07.10.026	No	NA	NA	1.27
22	07.10.173	No	NA	NA	3.48

HBV: Hepatitis B virus.

Precore/core promoter mutations and HBV viral load and ALT

Precore and core promoter mutations correlated with different levels of HBV DNA in HBeAg(+) and HBeAg(-) patients. In HBeAg(+) patients, the T1762/A1764 core promoter mutation correlated with lower HBV DNA levels ($P < 0.001$) (Table 6). The A1899 mutation was not associated with HBV DNA level ($P = 0.609$) and, as expected, no A1896 mutations were detected in the samples. The presence of either the T1762/A1764 or A1896 mutations also correlated with lower HBV DNA levels ($P = 0.011$). On the other hand, in HBeAg(-) patients, T1762/A1764 core promoter, as well as A1896 and A1899 precore mutations were not individually correlated with higher or lower HBV DNA level ($P = 0.095$, 0.231, 0.382, respectively), however, the presence of either the T1762/A1764 muta-

Table 6 Precore and core promoter mutations related to serum hepatitis B virus DNA and alanine aminotransferase in hepatitis B e antigen (+) patients

	Serum HBV DNA (log_{10} IU/mL) (mean ± SD) (*n*)		*P*-value	ALT (IU/L) [median (min-max)] (*n*)		*P*-value
	Absent	Present		Absent	Present	
T1762/A1764[1]	7.14 ± 1.46 (42)	5.60 ± 1.99 (29)	< 0.001	43.5 (13.0-748.0) (42)	48.5 (1.0-215.0) (29)	0.806
A1896[2]	6.98 ± 1.27 (48)	-	-	47.0 (1.0-748.0) (48)	-	-
A1899[2]	7.01 ± 1.25 (43)	6.68 ± 1.53 (5)	0.609	45.5 (1.0-748.0) (43)	55.0 (36.0-92.0) (5)	0.627
T1762/A1764 and A1896[2]	6.98 ± 1.27 (48)	-	-	47.0 (1.0-748.0) (48)	-	-
T1762/A1764 or A1896[2]	7.33 ± 1.23 (31)	6.33 ± 1.08 (17)	0.011	50.0 (15.0-748.0) (31)	41.5 (1.0-117.0) (17)	0.459

[1]Total samples hepatitis B e antigen (HBeAg) (+) *n* = 71; [2]Total samples HBeAg(+) *n* = 48. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

Table 7 Precore and core promoter mutations related to serum hepatitis B virus DNA and alanine aminotransferase in hepatitis B e antigen (-) patients

	Serum HBV DNA (log_{10} IU/mL) (mean ± SD) (*n*)		*P*-value	ALT (IU/L) [median (min-max)] (*n*)		*P*-value
	Absent	Present		Absent	Present	
T1762/A1764[1]	4.64 ± 2.00 (48)	5.23 ± 1.79 (60)	0.095	57.0 (6.0-302) (48)	51.5 (6.0-860.0) (60)	0.885
A1896[2]	5.44 ± 1.66 (17)	5.91 ± 1.35 (41)	0.231	46.0 (15.0-860.0) (17)	63.5 (14.0-302.0) (41)	0.321
A1899[2]	5.80 ± 1.29 (38)	5.72 ± 1.76 (20)	0.382	52.0 (14.0-860.0) (38)	58.5 (17.0-302.0) (20)	0.693
T1762/A1764 and A1896[2]	5.59 ± 1.51 (40)	6.17 ± 1.27 (18)	0.138	65.0 (14.0-860.0) (40)	51.0 (17.0-174.0) (18)	0.543
T1762/A1764 or A1896[2]	2.77 ± 4.31 (2)	5.88 ± 1.23 (56)	< 0.001	59.5 (23.0-96.0) (2)	52.0 (42.0-860.0) (56)	0.847

[1]Total samples hepatitis B e antigen (HBeAg) (-) *n* = 108; [2]Total samples HBeAg(-) *n* = 58. HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

tion or the A1896 mutation was associated with increased HBV DNA levels ($P < 0.001$) (Table 7). In addition, no correlations between precore and core mutations and serum ALT levels were observed in either the HBeAg(+) or HBeAg(-) patients (Tables 6 and 7).

DISCUSSION

The present study was an epidemiological investigation of the precore and core promoter mutations and their relationship to HBeAg expression levels in Indonesian patients. The majority of patients enrolled in this study were infected with HBV genotype B (73.7%), while the rest were genotype C (26.3%) (Table 1), which is consistent with previous reports[17,19-21]. The prevalence of HBeAg(-) chronic hepatitis B patients was 60.3%, which is similar to other Asian countries[16]. Among the HBeAg(-) patients, 79.6% were HBeAb(+), which meant that they were HBeAg seroconverted (Table 3). Studies in Europe, Asia, and the United States have all reported an increased prevalence of HBeAg(-) chronic hepatitis among HBsAg(+) patients[16]. Our results support previous studies which reported that HBeAg(-) chronic hepatitis B is the most common form of chronic HBV infection in Asia. However, in our study, most of the HBeAg(-) patients had been seroconverted, and the percentage of HBeAg(-) due to abolition of HBeAg synthesis was relatively low (20.4%).

Analysis of the A1896 mutation by direct sequencing demonstrated that all HBeAg(+) samples were wild type (i.e. did not bear the A1986 mutation) (Table 4), however, in our initial sequencing the A1896 mutation was found in three HBeAg(+) samples. The PCR fragments from these samples were cloned into pBluescript Ⅱ SK(+), and sequence analysis of ten clones showed that wild type virus was also present in some isolates. Thus, the presence of these wild type viruses was presumably responsible for HBeAg synthesis. On the other hand, a high percentage of the HBeAg(-) patients bore the A1896 precore mutation. As expected, in HBeAg(-) patients, this mutation was more prevalent in HBeAg non-expressing patients (87.7%) compared to seroconverted patients (65.1%) (Table 4). Nevertheless, the percentage of this mutation in seroconverted patients was relatively high. Since the A1896 mutation creates a premature stop codon which results in abolition of HBeAg synthesis[8-10], seroconverted patients cannot have always had virus with the A1896 mutation. It is believed that precore mutants emerge as a result of selection under immune pressure during the process of HBeAg seroconversion[22-24]. Therefore, at the early stage of infection, the virus might be a wild-type and the A1896 mutation occurs during the process of HBeAg seroconversion. In this study, because the mutation analysis was carried out after HBeAg seroconversion, it is also possible that the A1896 mutation detected in the samples occurred during the process of HBeAg seroconversion. In addition, the prevalence of the A1899 precore mutation was significantly higher in HBeAg(-) than in HBeAg(+) patients ($P = 0.004$), and was also higher in samples before HBeAg seroconversion compared to that after HBeAg seroconversion ($P = 0.003$) (Table 4). These results suggest that the A1899 mutation is associated with expression and seroconversion of HBeAg, which is in accordance with previous studies in Taiwanese patients[25]. Another study from Korea also reported that the A1899 mutation

was frequently found in HBeAg(-) patients, however, the authors found that the A1899 mutation was always accompanied by A1896 mutation[26], which was different to our results.

The frequency of the T1762/A1764 mutation was relatively high both in HBeAg(+) and HBeAg(-) patients, and there was no significant different between the two groups or between patients before and after HBeAg seroconversion. These results suggest that there is no independent association between the T1762/A1764 mutation on HBeAg expression and seroconversion. The presence of both the T1762/A1764 and A1896 mutations correlated with HBeAg expression and seroconversion, but was likely due to the effect of A1896 mutation alone. Although previous studies demonstrated that the core promoter region regulates transcription of the pregenomic and precore RNA and T1762/A1764 mutation suppression, it does not abolish the synthesis of HBeAg, leading to a reduction in HBeAg expression[12,27-29], and we did not observe this phenomenon in our study. Moreover, by measuring HBeAg titer quantitatively, a recent study also reported that the T1762/A1764 mutation reduced the expression of HBeAg[30]. In this study, however, HBeAg analysis was performed qualitatively, which perhaps explains the different results from those of previous studies.

Further analysis of precore and core promoter mutations in HBeAg non-expressed patients revealed that the A1896 mutation alone did not abolish HBeAg synthesis. Theoretically, A1896 mutation creates a premature stop codon which results in abolishment of HBeAg synthesis, however, A1896 was not found in all HBeAg non-expressed patients. Among twenty-two patients with HBeAg(-) and HBeAb(-), we identified the A1896 mutation in fifteen patients of which two did not show the A1896 mutation, but showed the T1762/A1764 mutation (Table 5). To confirm the A1896 mutation in these two samples, we cloned the amplified fragments into the plasmid, and sequenced eighteen clones, and no mutations were found in any of the clones. Furthermore, the A1899 mutation was not found in these samples. These results suggest that the expression of HBAg might not be affected by the A1896 mutation alone, however, further study is needed to investigate other factors involved in HBeAg expression.

In addition to precore and core promoter mutation, HBV variants with point mutations around the Kozak sequence (nucleotides 1809-1812) were also analyzed. Mutations at the Kozak sequence were less common in our samples and were not associated with HBeAg expression (data not shown), which is similar to the results from Korea[31]. Some studies have found that mutations in the Kozak sequence are correlated with HBeAg expression[32-34]. Our study demonstrated that these mutations do not play an important role in the clinical outcome of chronic hepatitis B patients. The differences may be attributable to genetic differences.

Another interesting finding of this study is that the effect of precore and core promoter mutation on HBV viral load was different in HBeAg(+) and HBeAg(-) patients. In HBeAg(+) patients, T1762/A1764 mutation was associated with lower viral load. On the other hand, this mutation was associated with higher viral load in HBeAg(-) patients, including HBeAg seroconverted patients. These results are in accordance with a previous study in Chinese patients[35,36]. It may be postulated that, among individuals who are HBeAg(+), those with both wild-type and mutant viruses have them in different phases of the infection, the former being in the first phase and the latter, at the end of the second phase. These patients experience different immune pressures, resulting in different levels of virus replication. However, those who are HBeAg(-), regardless of core promoter sequence, are in the same phase (the third phase) and experience similar immune pressures, resulting in similar levels of virus replication. If this is the case, it is not difficult to understand why T1762/A1764 core promoter mutations are associated with lower viral loads in HBeAg(+) patients, but have no effect in HBeAg(-) patients.

In conclusion, the percentage of HBV infected HBeAg (-) patients is relatively high in Indonesia. Most of the HBeAg(-) patients had been seroconverted and the remaining patients did not express HBeAg. A1896 mutation in the precore region was the major cause of the loss of HBeAg expression. T1762/A1764 core promoter mutations are associated with lower viral loads in HBeAg(+) patients, but are associated with higher viral loads in HBeAg(-) patients.

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COMMENTS

Background

Seroconversion from hepatitis B e antigen (HBeAg)-positive to hepatitis B e antibody (HBeAb)-positive correlates with reduced hepatitis B virus (HBV) replication in the liver and low infectivity during the natural course of infection. However, the immune pressure associated with HBeAg seroconversion selects for HBV variants that express little or no HBeAg. Although the patient may develop HBeAb, active HBV DNA replication continues with associated liver damage and is known as HBeAg-negative chronic hepatitis B. The aims of the study were to identify the prevalence of HBeAg-negative patients and to assess the association between HBV core promoter mutations and viral load in Indonesian patients.

Research frontiers

To date, there have been no reports on the prevalence of HBeAg-negative chronic hepatitis B in Indonesia. Therefore, it is important to obtain information on the HBeAg status of liver disease patients in Indonesia, and its association with precore and core promoter mutations. In addition, the correlation between precore and core promoter mutations and HBV replication is crucial.

Innovations and breakthroughs

The present study showed that the prevalence of HBeAg-negative chronic hepatitis B in Indonesia is high and most patients had seroconverted. The A1896 mutation was most likely to be the major cause of HBeAg loss. A1899 mutation is also associated with HBeAg-negative and is not always accompa-

nied by A1896 mutation. Furthermore, A1896 was not found in all HBeAg non-expressing patients, two HBeAg(-) and HBeAb(-) patients did not show either the A1896 or A1899 mutation, but had the T1762/A1764 mutation, suggesting that the expression of HBeAg might not be affected by the A1896 mutation alone. Interestingly, the T1762/A1764 mutation was associated with lower viral loads in HBeAg-positive, but not in HBeAg-negative patients.

Applications

The A1896 mutation can be used to predict HBeAg-negative variants in patients who achieve HBeAg seroconversion during disease progression or anti-viral therapy. In addition, the A1899 and T1762/A1764 mutations can be used to predict HBeAg-negative chronic hepatitis B, although there is no A1896 mutation.

Terminology

Core promoter: Part of the HBx gene that regulate the HBe and core gene expression. HBeAg seroconversion: The clearance of HBeAg by the production of HBeAb.

Peer review

The reported work is very intriguing and represents a large undertaking.

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ORIGINAL ARTICLE

Glutamine depletion induces murine neonatal melena with increased apoptosis of the intestinal epithelium

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Abstract

AIM: To investigate the possible biological outcome and effect of glutamine depletion in neonatal mice and rodent intestinal epithelial cells.

METHODS: We developed three kinds of artificial milk with different amounts of glutamine; Complete amino acid milk (CAM), which is based on maternal mouse milk, glutamine-depleted milk (GDM), and glutamine-rich milk (GRM). GRM contains three-fold more glutamine than CAM. Eighty-seven newborn mice were divided into three groups and were fed with either of CAM, GDM, or GRM *via* a recently improved nipple-bottle system for seven days. After the feeding period, the mice were subjected to macroscopic and microscopic observations by immunohistochemistry for 5-bromo-2'-deoxyuridine (BrdU) and Ki-67 as markers of cell proliferation, and for cleaved-caspase-3 as a marker of apoptosis. Moreover, IEC6 rat intestinal epithelial cells were cultured in different concentrations of glutamine and were subject to a 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate cell proliferation assay, flow cytometry, and western blotting to examine the biological effect of glutamine on cell growth and apoptosis.

RESULTS: During the feeding period, we found colonic hemorrhage in six of 28 GDM-fed mice (21.4%), but not in the GRM-fed mice, with no differences in body weight gain between each group. Microscopic examination showed destruction of microvilli and the disappearance of glycocalyx of the intestinal wall in the colon epithelial tissues taken from GDM-fed mice. Intake of GDM reduced BrdU incorporation (the average percentage of BrdU-positive staining; GRM: 13.8%, CAM: 10.7%, GDM: 1.14%, GRM *vs* GDM: $P < 0.001$, CAM *vs* GDM: $P < 0.001$) and Ki-67 labeling index (the average percentage of Ki-67-positive staining; GRM: 24.5%, CAM: 22.4% GDM: 19.4%, GRM *vs* GDM: $P = 0.001$, CAM *vs* GDM: $P =$

0.049), suggesting that glutamine depletion inhibited cell proliferation of intestinal epithelial cells. Glutamine deprivation further caused the deformation of the nuclear membrane and the plasma membrane, accompanied by chromatin degeneration and an absence of fat droplets from the colonic epithelia, indicating that the cells underwent apoptosis. Moreover, immunohistochemical analysis revealed the appearance of cleaved caspase-3 in colonic epithelial cells of GDM-fed mice. Finally, when IEC6 rat intestinal epithelial cells were cultured without glutamine, cell proliferation was significantly suppressed after 24 h (relative cell growth; 4 mmol/L: 100.0% ± 36.1%, 0 mmol/L: 25.3% ± 25.0%, $P < 0.05$), with severe cellular damage. The cells underwent apoptosis, accompanied by increased cell population in sub-G0 phase (4 mmol/L: 1.68%, 0.4 mmol/L: 1.35%, 0 mmol/L: 5.21%), where dying cells are supposed to accumulate.

CONCLUSION: Glutamine is an important alimentary component for the maintenance of intestinal mucosa. Glutamine deprivation can cause instability of the intestinal epithelial alignment by increased apoptosis.

Key words: Glutamine; Newborn mice; Artificial milk; Melena; Intestinal epithelial cells; Apoptosis

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INTRODUCTION

Amino acids have various roles in the living body; however, their cytobiological functions associated with cell proliferation or immune responses have not been fully elucidated. Among them, glutamine, which is the most abundant amino acid in the human body, has been recognized as a conditional essential amino acid in critical illness, stress, and injury[1-4]. Glutamine also has a key role in intermediary metabolism for rapidly dividing cells, such as enterocytes and cells of the immune system[1,5]. Indeed, the small intestine accounts for the largest uptake of glutamine of any organ, absorbing this amino acid from the lumen of the gut, as well as from the bloodstream[6]. Illness or injury can lead to a significant decrease in plasma levels of glutamine, and when this decrease is severe, it correlates with increased mortality[7,8]. Several studies have demonstrated the efficacy of either enteral or parenteral glutamine in adults and infants with a variety of conditions, such as bone marrow transplantation, critical illness, burns, trauma, surgically treated patients, and very low birth weight infants[9,10]. Thus, glutamine appears especially important for susceptible individuals who are in high stress conditions.

In the past, it was difficult to identify the roles played by these amino acids *in vivo*. Past studies involved the evaluation of immunological effects of amino acids by the administration of a diet rich or deficient in each amino acid (glutamine, arginine, *etc*.) by means of total parenteral nutrition or stomach tubing[11]. However, no such study in newborn mice has yet been reported. The lack of such a study in newborn mice is attributable to the fact that it is quite difficult to develop a method of alimentation or to prepare a diet for neonatal mice. To overcome these issues, Yajima *et al*[12] have recently developed artificial milk for mice with a composition very close to that of mouse maternal milk. Subsequently, artificial milk for mice with all of the proteins broken down into amino acids was developed. We expected that the use of this milk would make it possible for us to feed newborn mice with milk either deficient or enriched in certain amino acids. In addition, Hoshiba established and reported a new system for the artificial alimentation of rats and mice immediately after birth[13]. With this system, it is possible to administer certain nutrients orally to newborn mice and rats and to evaluate the effects of these nutrients directly.

In this study, we utilized the above improved special milk and feeding device to achieve the following scientific aims: (1) to explore what happens when newborn mice are fed with glutamine-devoid milk; and (2) to dissect the physiological mechanism of glutamine deprivation-oriented events.

MATERIALS AND METHODS

Bottle-nipple system

The nursing bottle and nipple, which were made by Hoshiba[13], were used to feed the newborn mice. The nursing bottle has three tubes (a filling tube, an outlet tube, and a ventilation tube) and the nipples are made of a silicone rubber. The nipple consists of inner and outer parts, and a stopper devised to control the pressure as well as to avoid milk leakage.

Animals and study design

Specific-pathogen-free Jcl:ICR pregnant mice were purchased from Charles River Laboratories (Yokohama, Japan). They were maintained under the following environmental conditions: lighting, 12:12-h light:dark cycle; temperature, 22 to 25℃; air changes, 12 to 14 times per hour; and humidity, 40% to 50%. Newborn pups were separated from each dam immediately after birth. They were reared with artificial milk from the nursing bottle four times per day (09:00, 12:30, 16:00, and 20:00) for seven days and sacrificed on day eight. Body weights of the pups were measured before and after feeding, and the difference between them was represented the quantity of feeding.

Table 1 Amino acid components of complete amino acid milk

Amino acid	mg/100 mL of milk[1]
Valine	0.60 ± 0.02
Leucine	1.06 ± 0.04
Isoleucine	0.48 ± 0.04
Lysine	0.88 ± 0.06
Threonine	0.47 ± 0.02
Methionine	0.34 ± 0.04
Histidine	0.25 ± 0.03
Phenylalanine	0.51 ± 0.16
Tryptophan	0.19 ± 0.01
Alanine	0.40 ± 0.00
Arginine	0.32 ± 0.01
Glutamine	2.09 ± 0.20
Proline	0.74 ± 0.15
Cystine	0.08 ± 0.00
Tyrosine	0.56 ± 0.05
Asparagine	0.91 ± 0.07
Glycine	0.19 ± 0.00
Serine	0.53 ± 0.01

[1]The amount of each amino acid is presented as average ± SE (mg/100 mL of milk).

Milk formula

Artificial milk was purchased from Meiji Dairies Corporation (Odawara, Japan). The amino acid composition of the milk is shown in Table 1. The composition of the milk was made as similar as possible to mouse maternal milk. Table 2 shows the composition of mouse milk and artificial amino acid milk. The artificial amino acid milk showed an extremely high osmotic pressure compared to that of mouse maternal milk, because the proteins in the milk used in this study were in the form of amino acids with a low amount of fat. Thus, 10% lipid microsphere Intralipid (Nihon Pharmaceutical Co. Ltd., Tokyo, Japan) was added to this artificial amino acid milk at a ratio of 1:1 to yield the complete amino acid milk, thus the amount of amino acid contained in this milk was 50% of the initial amount with 16% fat, similar with that of mouse milk (22%). We named this milk complete amino acid milk (CAM). Furthermore, glutamine rich milk (GRM) had three-fold more glutamine than CAM, whereas glutamine-depleted milk (GDM) contained no glutamine.

Histological study

Resected tissues from the mice were fixed in 10% formalin, embedded in paraffin, and cut at a thickness of 5 μm. Sections were deparaffinized, rehydrated, and stained with hematoxylin and eosin. On day eight, the animals were perfused with 2% glutaraldehyde (5 mL) and fixed, followed by the removal of each organ for subsequent thin slicing and observation under an electron microscope.

Immunohistochemistry

To detect cells synthesizing DNA, 5-bromo-2'-deoxyuridine (BrdU) was injected (100 mg/kg i.p.) 1 h before sacrificing the animals. The organs were then placed in 4% paraformaldehyde for 48 h prior to paraffin embedding. BrdU incorporation in colon sections was determined by immunohistochemical staining. Briefly, sections were deparaffinized with xylene (Mallinckrodt Baker, Paris, Kentucky) and taken through a graded series of alcohol/water mixtures to rehydrate the tissue. To retrieve the antigen, sections were incubated with 2 mol/L HCl for 30 min and then incubated with 0.1 mol/L $Na_2VO_4O_7$ at room temperature for 30 s. Sections were exposed to rat anti-BrdU monoclonal antibodies (OBT, Oxford, UK) for 60 min at room temperature. Peroxidase-conjugated anti-mouse IgG_1 antibody (DAKO, Carpinteria, CA) was then applied, and 3,3'-diaminobenzidine chromogen was added as the peroxidase substrate. A light counterstain of modified Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan) was then applied so that unlabeled nuclei could be easily identified. Immunostaining for Ki-67 and cleaved caspase-3, the biological markers for cell proliferation and apoptosis, respectively, used a rabbit anti-Ki-67 monoclonal antibody (Ylem, Rome, Italy) and a rabbit anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA), respectively.

Cell culture and treatments

There was no commercially available mouse intestinal epithelial cell line; therefore, we used IEC6 rat intestinal epithelial cells, which were obtained from Dainippon Pharmaceutical Co. Ltd. (Tokyo, Japan), and maintained in dulbecco's modified eagle medium (DMEM) with 10% FBS and 4 mmol/L L-glutamine for 24 h. Cells were incubated in DMEM with 0, 0.4, or 4 mmol/L L-glutamine without FBS for the indicated time periods (0, 3, 6, 12, 24 and 48 h).

Proliferation assay

The number of surviving cells was measured by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay (Roche, Indianapolis, USA) for the indicated time periods after plating.

Immunoblot analysis of caspase-3

Cells were lysed in 1 × sodium dodecyl sulfate (SDS) sample buffer (62.5 mmol/L Tris-HCl (pH 6.8 at 25℃), 2% w/v SDS, 10% glycerol, 50 mmol/L dithiothreitol, and 0.01% w/v bromophenol blue or phenol red) and centrifuged at 4℃. The sample viscosity was reduced by pipetting. The samples were then boiled for 5 min and cooled on ice. The samples were separated by SDS-poly-acrylamide gel electrophoresis on 12% polyacrylamide gels, transferred onto a polyvinylidene difluoride membrane, immunoblotted with Rabbit anti-caspase-3 polyclonal antibody (Upstate Cell Signaling Solutions, Charlottesville, VA) and Rabbit anti-cleaved caspase-3 polyclonal antibody (Millipore corporation, Bedford, MA) followed by anti-rabbit immunoglobulin G-horseradish peroxidase (GE, Piscataway, NJ). Immunoreactive proteins were visualized using an Enhanced Chemi-Luminescence kit according to the manufacturer's protocol (GE, Piscataway, NJ).

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) staining. Briefly, IEC6 cells were first seeded into 10-cm

Table 2 Nutrient composition of mouse milk and artificial amino acid milk

	Mouse milk	Artificial amino acid milk	CAM[1]	GDM	GRM
Osmotic pressure (mOsm/kg)	300	1800	1161	ND	ND
Glutamine (g/L)	ND	24.23	12.12	0	36.35
Fat (%)	22	10	16	16	16

[1]Emulsified fat was added to artificial amino acid milk at a ratio of 1:1 to yield complete amino acid milk (CAM), because the proteins in the milk used in this study were in the form of amino acids, resulting in a very high osmotic pressure with a low amount of fat. As a result, the amount of amino acid contained in the CAM is 50% of the initial amount. GDM: Glutamine-deleted milk; GRM: Glutamine-rich milk; ND: Not determined.

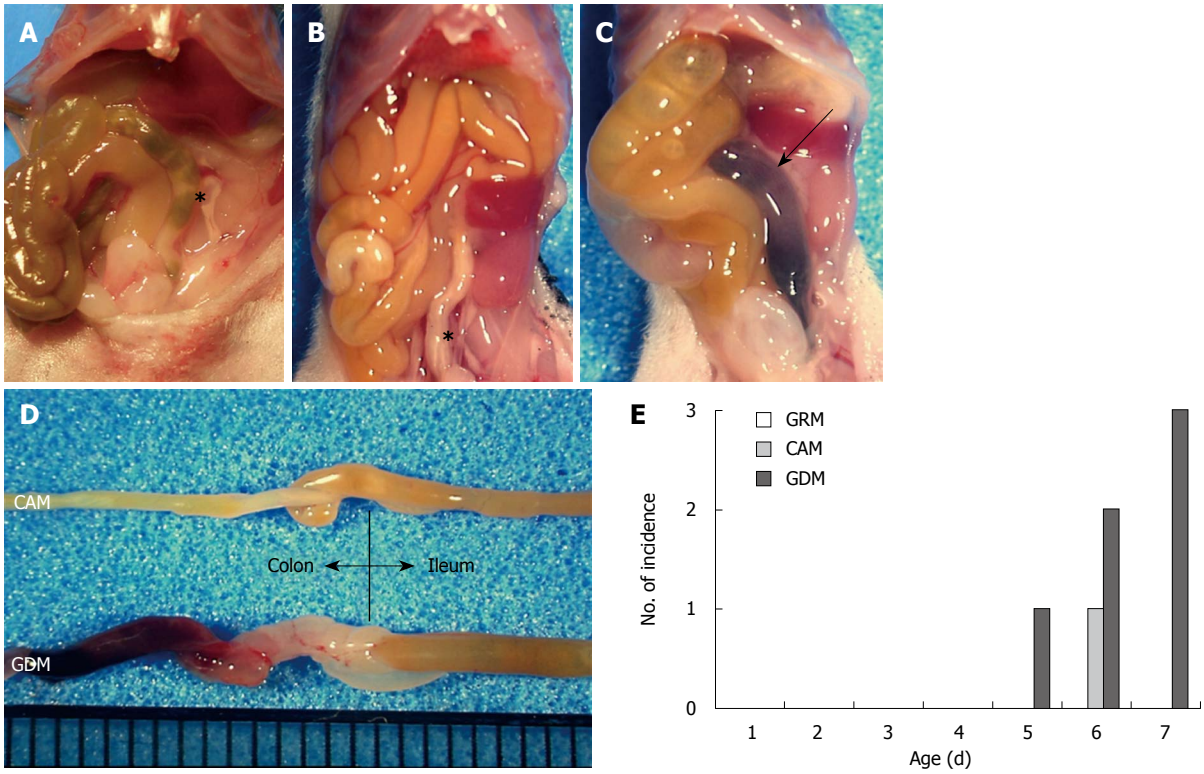

Figure 1 Macroscopic views of milk-fed mice with colonic hemorrhage. Representative macroscopic views of newborn mice that were fed with glutamine-rich milk (GRM) (A), complete amino acid milk (CAM) (B), and glutamine-deleted milk (GDM) (C) are shown. Compared to the colons of GRM-mice and CAM-mice (asterisks), those of the GDM-mice appeared distended and edematous, with a pool of blood (arrow); D: Close-up views of the resected intestines from a CAM-mouse and a GDM-mouse, shown for comparison; E: Bar chart of the number of mice with melena on each day.

dishes at a cell density of 5×10^5. After further culture for 48 h and 96 h, the cells were trypsinized and harvested in phosphate buffered saline (PBS) followed by resuspension in PBS at 1-2 $\times$ 10^6/mL. Finally, the cells were stained with 0.5 mL of PI staining solution (3.8 mmol/L sodium citrate, 50 mg/mL PI in PBS) for 1 h at room temperature to analyze cell cycle distribution by FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) excitation at 488-nm. The DNA-linked red fluorescence (PI) was measured through a 600-nm wavelength filter. This experiment was performed three times.

Statistical analysis

For *in vivo* experiments, the significance of differences between the control and test values was determined by Tukey's test using JMP 6.0.3 software (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

RESULTS

Mice fed with GDM display colonic hemorrhage

Newborn mice were assigned to three groups (GRM, CAM, and GDM) and were fed four times a day according to the above-mentioned schedule. During the observation periods, the mice gained weight regardless of the amount of glutamine, and there were no significant differences between the groups (data not shown).

We measured the glutamine concentration in serum taken from each mouse fed with different types of milk. As expected, the GRM-mouse serum contained the highest amount of glutamine (7.53% of total amino acids), while the GDM-mouse serum had the lowest amount of glutamine (4.92% of total amino acids) in circulation. The CAM-mice maintained less glutamine in serum (5.74% of total amino acids) than the dam-reared mice (6.44%).

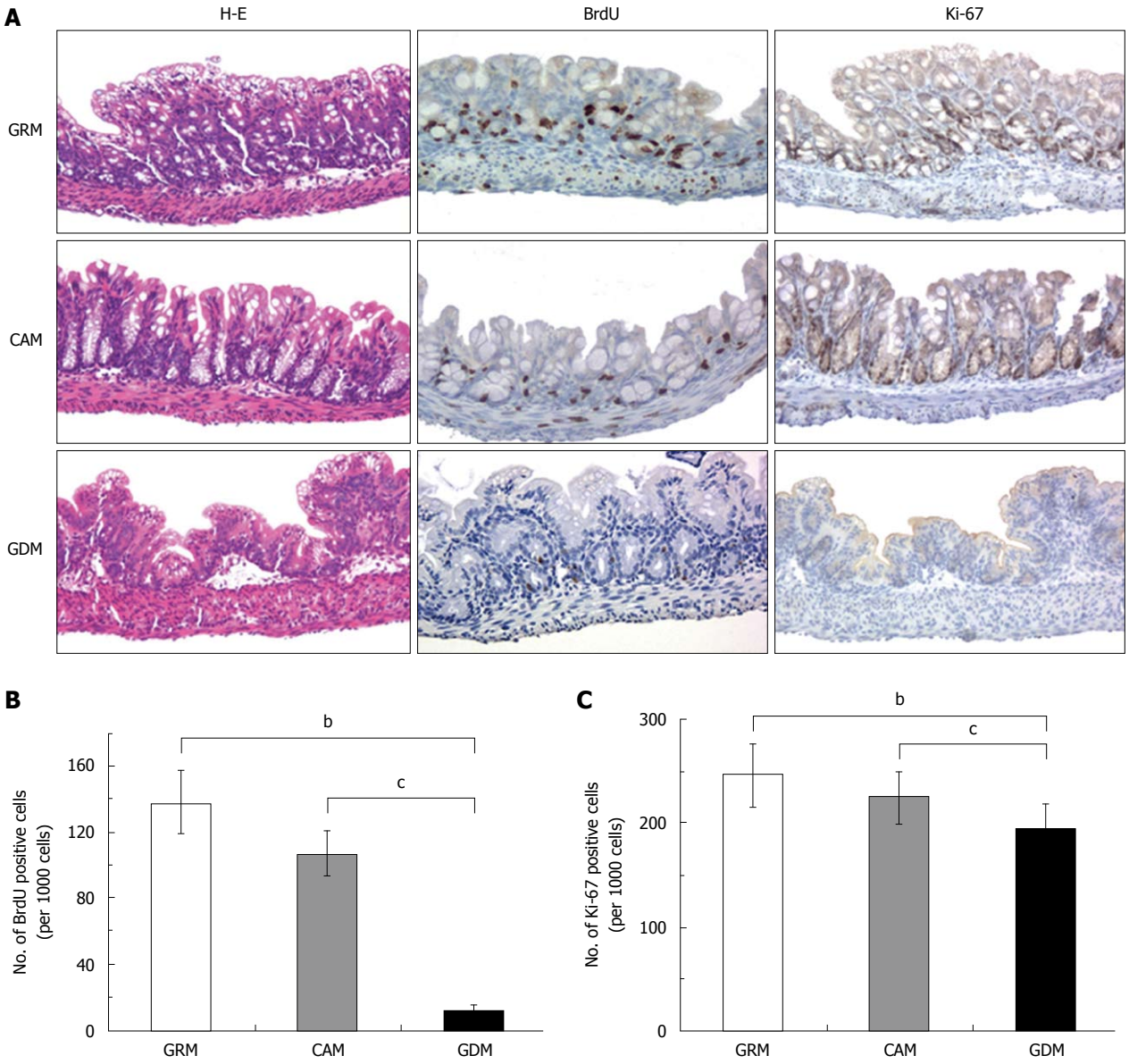

Figure 2 Glutamine depletion induces severe damage of the colonic epithelial structure with reduced epithelial cell growth. A: Representative pictures of H-E staining (left panels) and immunohistochemical staining for BrdU (middle panels) and Ki-67 (right panels). Each colonic epithelium was taken from infant mice fed with glutamine-rich milk (GRM), complete amino acid milk (CAM), or glutamine-deleted milk (GDM). Magnification, × 200. Positive stained epithelial cells with BrdU (B) and Ki-67 (C) were counted and compared with each group in a histogram. $^{b}P \leqslant 0.001$; $^{c}P < 0.05$.

These data suggested that the amount of glutamine in the milk fed to the pups did affect the concentration of glutamine circulating in the animal body.

When the mice were sacrificed on day eight, we found a pool of blood in the colons of the GDM-fed mice accompanied by melena (Figure 1A-C). In addition, the entire bowel of these mice showed a massive edematous change, accompanied by wall thickening, redness, and dilatation of the small intestine (Figure 1D). No macroscopic changes were observed in mice fed with glutamine-containing milk, except for one mouse fed with CAM (Figure 1A-D). These events were observed in six of 28 GDM-fed mice (21.4%) and one CAM-fed mouse (3.3%), and started on days five to seven (one mouse on day five, four mice including the mouse fed with CAM, on day six, and two mice on day seven) (Figure 1E). No mice fed with GRM developed melena during the observation period. The incidence of melena was significantly higher in the GDM group compared to the other groups (Figure 1E), suggesting that glutamine depletion must affect the maintenance of the neonatal gastrointestinal tract.

Glutamine depletion reduces cell proliferation and increases apoptosis in the colonic epithelium

Histological observation by hematoxylin-eosin staining revealed the destruction of the villous structure, wall thickening with edematous dilatation of the submucosal layer, and inflammatory cell infiltration around the hemorrhage site of the colons in the mice fed without glutamine (Figure 2A). In addition, a reduction of goblet cells was noted in this group, suggesting a disorder of cell maturation. Interestingly, the intestinal mucosa of the GRM-fed mice were

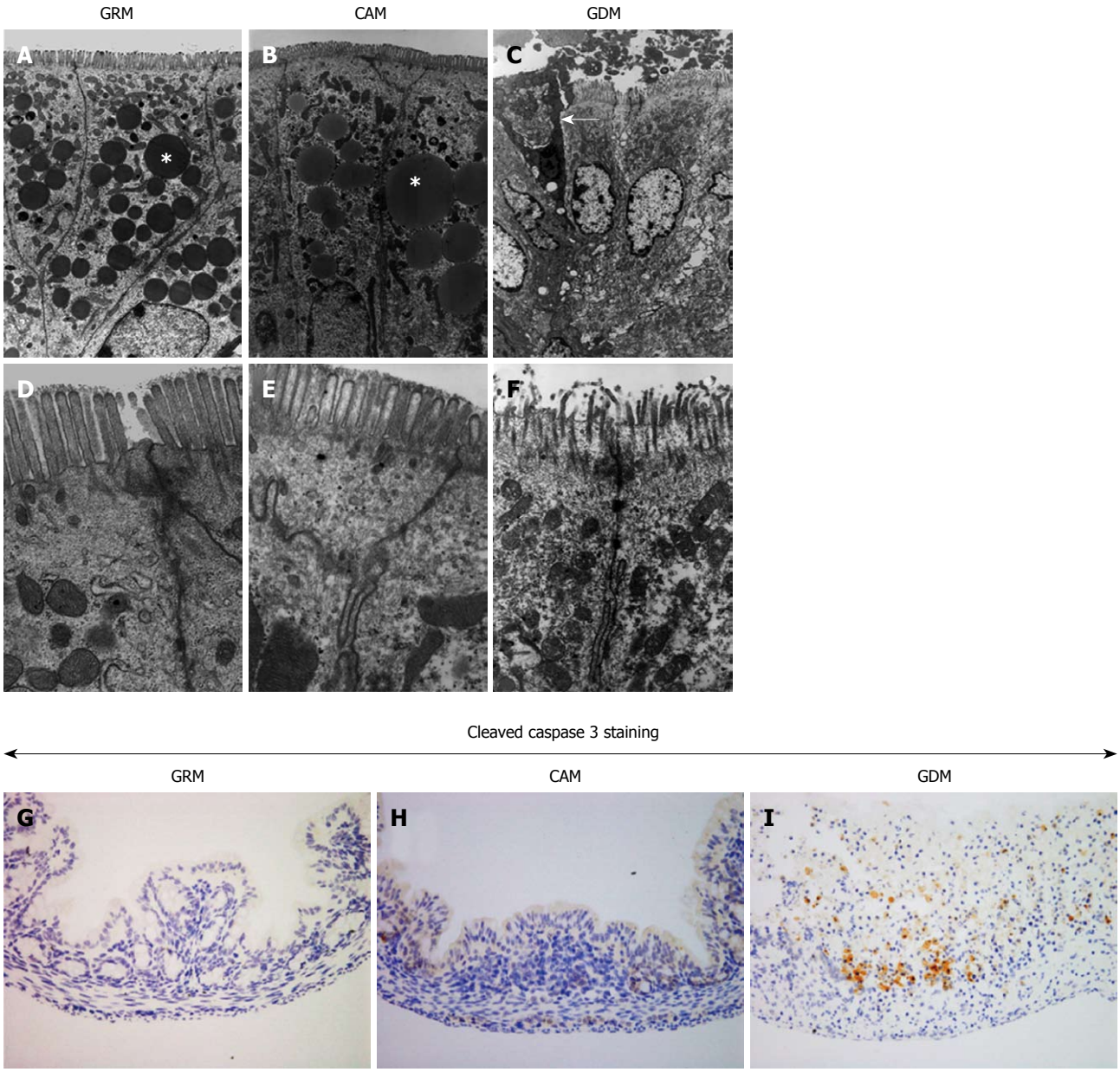

Figure 3 Apoptotic changes observed in the damaged colonic epithelium. Electron micrographs of colonic epithelia obtained from infant mice with glutamine-rich milk (GRM) (A, D), complete amino acid milk (CAM) (B, E), and glutamine-deleted milk (GDM) (C, F) at low magnification (A-C) and high magnification (D-F). Asterisks (*) represent lipid droplets and the arrow shows an apoptotic cell. G-I: Optical microscopic views (magnification, × 200) of immunohistochemistry for cleaved caspase-3 as a marker of apoptosis using resected tissues from the same mice (G: GRM, H: CAM, I: GDM).

higher than those of the CAM-fed mice (Figure 2A).

We then assessed cell proliferation in colonic epithelial cells by immunostaining for BrdU and Ki-67, which are markers for DNA synthesis and cell proliferation, respectively (Figure 2A). BrdU incorporation was significantly decreased in colonic epithelial cells of the GDM-fed mice compared to those of CAM-mice and GRM-mice (average percentage of BrdU-positive staining per 1000 cells; GRM: 13.8%, CAM: 10.7%, GDM: 1.14%, Tukey test; GRM *vs* GDM, $P < 0.001$; CAM *vs* GDM, $P < 0.001$) (Figure 2B). Ki-67-positive staining was also significantly decreased in the colonic epithelia of the GDM-fed mice (average percentage of Ki-67-positive staining; GRM: 24.5%, CAM: 22.4%, GDM: 19.4%, Tukey test; GRM *vs* GDM, $P = 0.001$; CAM *vs* GDM, $P = 0.049$) (Figure 2C). These data indicated that glutamine deprivation strongly diminished cell growth of the intestinal epithelium.

We further examined the damaged colonic mucosa under the electron microscope. Figure 3A-F shows representative pictures of colonic epithelia from each group. Glutamine deprivation caused deformation of the nuclear membrane and the plasma membrane, accompanied by the destruction of microvilli and the disappearance of glycocalyx, resulting in nuclear deformation and chromatin degeneration. Fat droplets, which were seen in the colons of the mice fed with the glutamine-containing milk, were absent in the GDM Group. Furthermore, a partial loss of colonic epithelial cells was noted in the GDM Group, indicating that glutamine deprivation promotes cell death. To confirm glutamine deprivation-induced cell death,

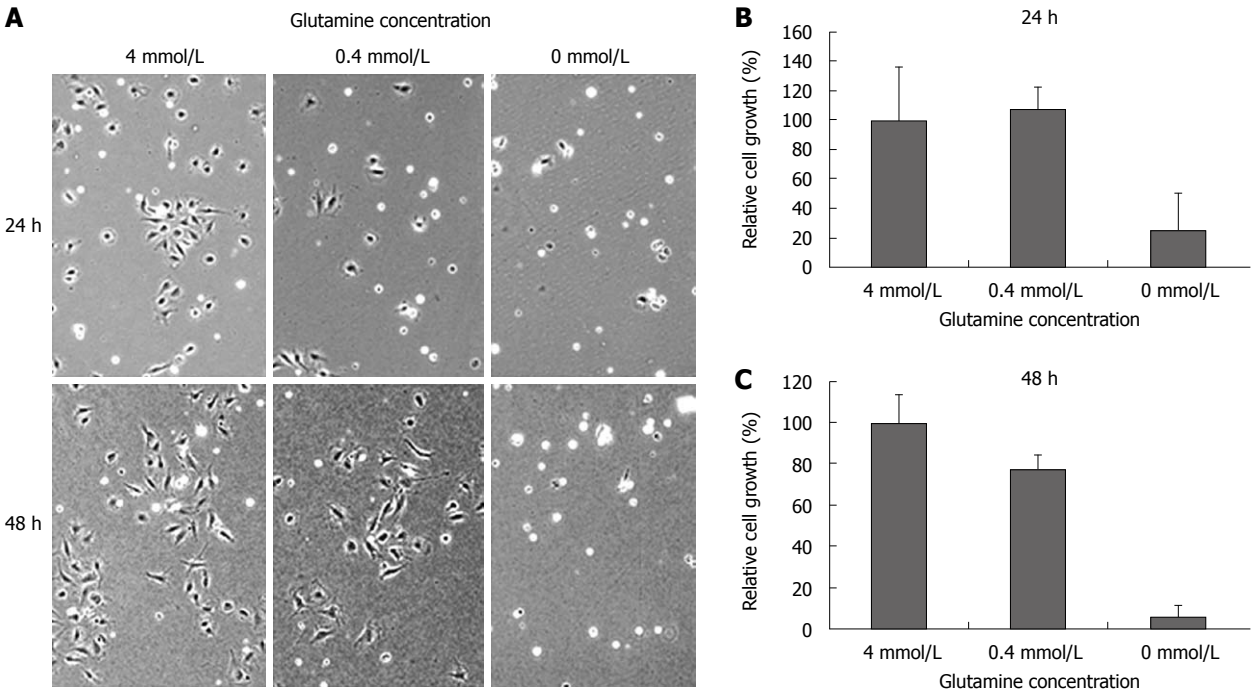

Figure 4 Glutamine depletion suppresses cell proliferation of cultured intestinal epithelial cells. A: IEC6 rat intestinal epithelial cells were treated with media containing different amounts of glutamine (0, 0.4 and 4 mmol/L); Cell morphology and cell number were observed at the indicated time points (B: 24 h, C: 48 h).

cleavage of caspase-3 was assessed by immunohistochemistry. As shown in Figure 3G-I, there was a remarkable number of cleaved caspase-3-positive stained cells in the glutamine-deprived mucosa, suggesting that a lack of glutamine induces colonic epithelial cell death, possibly through the process of apoptosis.

Glutamine depletion reduces cell proliferation in IEC6 rat intestinal cells

To clarify the effect of glutamine depletion on intestinal epithelial cell growth, IEC6 rat intestinal epithelial cells were cultured in the presence of varying concentrations of glutamine. Microscopic observation showed that glutamine depletion damaged cell morphology and reduced cell density (Figure 4A). As shown in Figure 4B and C, glutamine depleted conditions significantly suppressed IEC6 cell growth after 24 h (4 mmol/L: 100.0 ± 36.1, 0 mmol/L: 25.3 ± 25.0, $P < 0.05$), while there was no apparent difference between 4 mmol/L and 0.4 mmol/L after 24 h (4 mmol/L: 100.0 ± 36.1, 0.4 mmol/L: 107.4 ± 15.4, $P = 0.7614$), or after 48 h (4 mmol/L: 100.0 ± 14.0, 0.4 mmol/L: 77.4 ± 6.9, $P = 0.0659$). We further assessed the inhibitory effect on cell proliferation by cell cycle analysis, using flow cytometry (Figure 5A and B). We did not observe significant cell growth arrest at the G1 or G2 phase; however, an increased cell population at the sub-G0 phase was observed within 48 h of glutamine depletion (4 mmol/L: 1.68%, 0.4 mmol/L: 1.35%, 0 mmol/L: 5.21%, Figure 5B), which is consistent with the observation in the animal model that glutamine-depletion seemed to be lethal to intestinal epithelial cells.

Finally, we determined whether glutamine depletion-mediated cell death in cultured IEC6 cells occurs due to induction of apoptosis. Cells cultured with a complete depletion of glutamine showed a time-dependent decrease in caspase-3 expression, accompanied by cleavage of caspase-3 within 24 h (Figure 5C). These data support our finding in the animal study that a lack of glutamine affects the maintenance of intestinal epithelial cells, suppresses cell growth and induces apoptosis, resulting in melena.

DISCUSSION

Until recently, it was quite difficult to study the physiological and cytobiological effects of amino acids *in vivo*, especially for neonatal animals, because of the lack of methods of alimentation or of preparing a diet for neonatal mice. In this study, using a totally new milk feeding system and amino acid milk, we successfully observed the physiological effects of glutamine depletion in newborn mice. Interestingly, a significantly high incidence of colonic hemorrhage occurred in mice fed with GDM, compared to the CAM or GRM groups. We had one CAM-fed mouse that had a similar colonic hemorrhage on day six. This mouse might have had a genetic/physiological susceptibility to the event. It could also have been caused by the fact that the amount of amino acid contained in the CAM was 50% of the ordinary amount because emulsified fat was added to the artificial amino acid milk at a ratio of 1:1 to yield CAM. The proteins in the milk used in this study were in the form of amino acids, resulting in a very high osmotic pressure with a low amount of fat (Table 2). If the artificial amino acid milk is applied directly to newborn mice without adding fat, which is a major nutrient for them, a fat deficiency might occur. Therefore, the melena that occurred in the CAM-

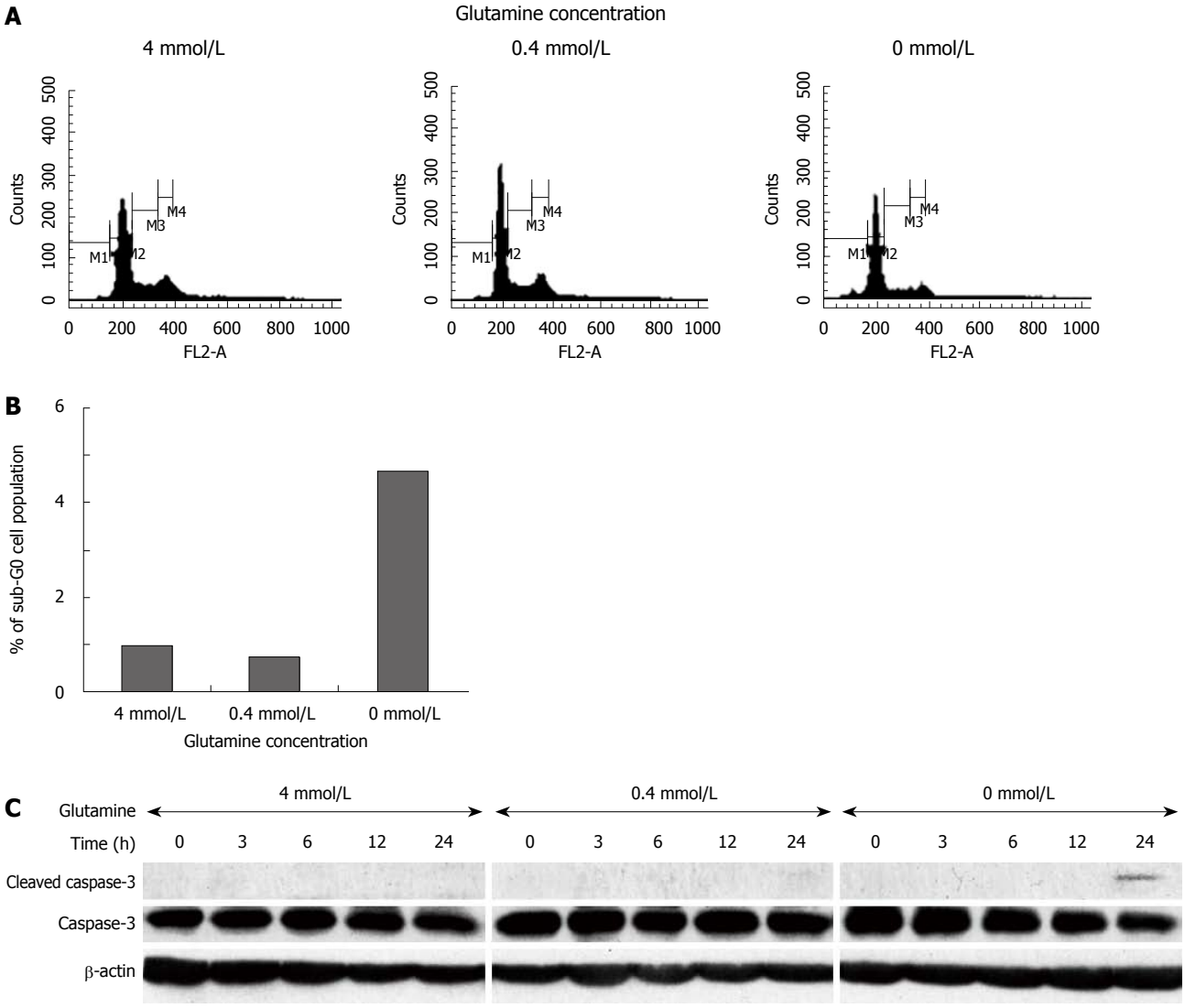

Figure 5 Potent antiproliferative effect of glutamine depletion results in increased cleavage of caspase-3. Cell cycle distribution under culture condition with different concentrations of glutamine was analyzed by flow cytometry (A) and cell populations at the sub-G0 phase were compared with each other (B); C: Immunoblotting for caspase-3 and cleaved caspase-3 revealed the induction of apoptosis in IEC6 cells after 24 h of culture without glutamine. Each experiment was independently repeated three times and the representative data among the similar results are shown.

fed mouse may be explained by an insufficient intake of glutamine. Meanwhile, no animal developed colonic hemorrhage in the GRM Group; thus, we assume that the glutamine level in this milk was high enough to maintain the intestinal epithelium.

Other macroscopic findings in the hemorrhagic intestines of the GDM-fed mice were the apparent inflammatory changes of the entire intestine, with intestinal wall thickening by edema. Microscopic observations supported these findings, with infiltration of inflammatory cells in and around destroyed colonic mucosa at the site of the hemorrhage. On the other hand, the height of the colonic mucosa of GRM-fed mice was well conserved and was higher than that of the CAM mice. In addition, as more glutamine was administered, more positive-staining cells for BrdU and Ki-67 appeared, suggesting that glutamine is a critical nutrient for the proliferation of intestinal cells. Moreover, both electron microscopic observations and immunohistochemistry for cleaved caspase-3 reflected an increased incidence of apoptosis induced by glutamine depletion. However, no exact intracellular mechanism has been identified for the destruction of the nuclear membrane and microvilli, or the disappearance of glycocalyx, which were induced by GDM. Clarifying the molecular/biophysical mechanism of this glutamine depletion-mediated event will be crucial to understanding the intrinsic functions of glutamine or other amino acids.

Experimental data from cultured IEC6 cells supported the findings of the animal experiments, with the reduced cell proliferation, accumulation of a cell population in the sub-G0 phase, and the cleavage of caspase-3 under glutamine-depleted culture conditions. According to these results, glutamine depletion induces cell death of colonic mucosa, presumably due to an acute induction of apoptosis, followed by the destruction of mucosa maintenance, which eventually leads to colonic hemorrhage.

In the present study, it was remarkable that colonic hemorrhage could be induced simply by removing one

amino acid, glutamine. Glutamine has attracted close attention as an amino acid nutrient and as an immunopotentiating factor for intestinal cells[14-16]. However, no case of intestinal hemorrhage induced by glutamine deficiency has been reported to date. Although glutamine is a non-essential amino acid and can be produced in the living body, neonatal mice are actively growing and are very dependent on external alimentation due to a heavier consumption of nutrients, including glutamine, compared to adults[17]. It seems likely that the consumption of glutamine by these neonatal mice might exceed the amount of glutamine pooled and formed in the living body, leading to the emergent status of glutamine deficiency.

In terms of molecular/biological effects of glutamine, there are outstanding several issues that remain to be clarified. One of our interests is to explore the possible involvement of certain signaling pathways. It has recently been unveiled that amino acids are involved in the control of the mTOR signaling pathway[18]. We have also demonstrated previously that leucine activates the mTOR signaling pathway in a hepatocellular carcinoma cell line[19]. It has also been shown that leucine and arginine activate the mTOR signaling pathway in a small bowel epithelial cell line derived from rats[20]. We also found that glutamine regulates the activation of this pathway in the same cell line[21]. Therefore, future studies should focus on the mechanism for the induction of colonic hemorrhage and how glutamine is involved in the intracellular signaling pathway, such as in mTOR[22-24].

In conclusion, we found that feeding neonatal mice with GDM induced a high incidence of colonic hemorrhage within a week, and that this was due to an induction of epithelial cell death. Further investigation is necessary to explore the biological mechanism.

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COMMENTS

Background

Glutamine, the most abundant amino acid in the human body, is conditionally essential, especially for susceptible individuals who are in high stress conditions. A lack of glutamine correlates with increased mortality.

Research frontiers

The small intestine accounts for the largest uptake of glutamine of any organ, absorbing this amino acid from the lumen of the gut, as well as from the bloodstream. Past studies involved the evaluation of immunological effects of amino acids by the administration of a diet rich or deficient in each amino acid (glutamine, arginine, *etc.*) by means of total parenteral nutrition or stomach tubing. However, no such study in newborn mice has yet been reported. The lack of such a study in newborn mice is attributable to the difficulty in developing a method of alimentation or preparing a diet for neonatal mice.

Innovations and breakthroughs

The authors developed three kinds of artificial milk with different amounts of glutamine. Using these amino acid milks and a recently improved nipple-bottle feeding system, they successfully fed the newborn mice with the new amino acid milk and observed colonic hemorrhage in mice fed without glutamine. It was remarkable that colonic hemorrhage could be induced simply by removing one amino acid, glutamine. In addition, glutamine deprivation can cause instability of intestinal epithelial alignment by increased apoptosis.

Applications

No exact intracellular mechanism has been identified for the destruction of the nuclear membrane and microvilli or the disappearance of glycocalyx, which were induced by glutamine depletion. Thus, we must explore the mechanism of the induction of colonic hemorrhage and investigate glutamine's involvement in the intracellular signaling pathway, to better understand the intrinsic functions of glutamine and other amino acids.

Peer review

The authors have described that glutamine depletion induces neonatal mice melena and apoptosis of intestinal epithelium *in vivo* and *in vitro*. The authors obtained good data allowing the conclusion that glutamine depletion induces neonatal mice melena and apoptosis of intestinal epithelium, using appropriate methods. An animal model was established for glutamine deprivation-induced destruction of the intestinal epithelium. It would also be meaningful to study the effects of glutamine-deprivation on human intestinal function.

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ORIGINAL ARTICLE

Prediction of gastric cancer metastasis through urinary metabolomic investigation using GC/MS

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Abstract

AIM: To gain new insights into tumor metabolism and to identify possible biomarkers with potential diagnostic values to predict tumor metastasis.

METHODS: Human gastric cancer SGC-7901 cells were implanted into 24 severe combined immune deficiency (SCID) mice, which were randomly divided into metastasis group (n = 8), non-metastasis group (n = 8), and normal group (n = 8). Urinary metabolomic information was obtained by gas chromatography/mass spectrometry (GC/MS).

RESULTS: There were significant metabolic differences among the three groups (t test, P < 0.05). Ten selected metabolites were different between normal and cancer groups (non-metastasis and metastasis groups), and seven metabolites were also different between non-metastasis and metastasis groups. Two diagnostic models for gastric cancer and metastasis were constructed respectively by the principal component analysis (PCA). These PCA models were confirmed by corresponding receiver operating characteristic analysis (area under the curve = 1.00).

CONCLUSION: The urinary metabolomic profile is different, and the selected metabolites might be instructive to clinical diagnosis or screening metastasis for gastric cancer.

Key words: Metabolomic profile; Gastric cancer; Metastasis; Biomarker; Gas chromatography/mass spectrometry

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INTRODUCTION

Gastric cancer is the second leading cause of cancer death worldwide, and in many Asian countries, such as China[1,2]. Until now, there has been no effective treatment for gastric cancer. Even among patients undergoing gastrectomy,

because of locoregional relapse and distant metastases, the 5-year survival rates remain disappointing[3]. Early dissemination of the disease through the lymphatic system, blood and peritoneum has limited the therapeutic effects of optimal surgery, except in patients with relatively early-stage tumors[4]. Therefore, it is significant to establish an accurate early diagnosis of gastric cancer. Currently, the diagnosis or screening of gastric cancer or tumor recurrence mainly depends on endoscopy and pathological examinations. The ratio for identifying early gastric cancer with endoscopy is higher than that with X-ray[5], and the diagnosis of gastric cancer using endoscopy is more accurate[6]. Nevertheless, the results of endoscopy are easily affected by artificial factors (e.g. the experience of the endoscopist). Over the past years, epidemiological data have shown that *Helicobacter pylor*i (*H. pylori*) infection is strongly associated with the development of gastric cancer[1], and *H. pylori* eradication may be considered as a strategy to prevent gastric cancer[7]. In addition, investigation of gastric cancer tissues and some biomarkers have been used for screening gastric cancer[8-13]. However, compared with tissues and serum, the markers acquired from urine are noninvasive and convenient, especially in the patients with recurrent gastric cancer. The urinary metabolic profiling could be used to get urinary metabolites as gastric cancer or tumor recurrence biomarkers.

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems[14], and offers an analysis of metabolite level changes in biological samples[15]. In recent years, studies of metabolomics used in various diseases have been conducted, such as stomach cancer[16], lung cancer[17], renal cancer[18,19], brain tumors[20], and colorectal cancer[21-24]. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are the most commonly employed techniques for measuring the metabolome[14]. MS-based techniques, including gas chromatography/mass spectrometry (GC/MS), GC-MS/MS, liquid chromatography/mass spectrometry (LC-MS) and LC-MS/MS, are among the most efficient and versatile for quantitative analysis of endogenous and exogenous substances in biological samples[25]. Because of its peak resolution, high sensitivity and reproducibility, GC/MS has been well established and widely utilized in metabolomics[26-28].

In this study, we have established a human gastric cancer non-metastasis model and a metastasis model using severe combined immune deficiency (SCID) mice, and deployed GC/MS following chemical derivatization to profile the mouse model urinary specimens and their matched urine. The metabolic differences among the three groups were characterized by principal components analysis (PCA). On the basis of its results, we expected that the potential metabolic biomarkers could be found in mice for early diagnosis and screening the metastasis or the recurrence of gastric cancer.

MATERIALS AND METHODS

Chemicals and materials

Tetrahydrofuran (THF) and bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Vacuum dryer was purchased from Shanghai NOTED Technologies. All other reagents were obtained from Sinopharm Chemical Reagent Co. Ltd.

Animal models

Male SCID mice were acquired from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animals used were 6-wk old and weighed 20-25 g. Animal and experimental procedures were performed according to the relative ethical regulations for the care and use of laboratory animals of our university. Human gastric cancer SGC-7901 (Shanghai Cancer Institute), a poorly-differentiated adenocarcinoma line, was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Tumors were cut out aseptically. Necrotic tissues were cut and the reserved healthy tumor tissues were scissor minced into pieces (about 3 mm × 4 mm in diameter) in Hank's balanced salt solution. Each tumor piece was weighed and adjusted to be approximately 100 mg. All animals were randomly divided into metastasis group (n = 8), non-metastasis group (n = 8), and normal group (n = 8). Animal models were made using orthotopic implantation of histologically intact tissue of human gastric cancer[29]. Mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision of the metastatic group and the normal group was made through the left upper abdominal pararectal line. Then peritoneal cavity was carefully exposed and a part of serosal membrane in the middle of the greater curvature of stomach was mechanically injured by scissors. A tumor piece of 100 mg was fixed on each injured site of the serosal surface of the metastatic group, while normal control mice received no tumor implantation. The stomach was then returned to the peritoneal cavity, and the abdominal wall and skin were closed. An incision of the non-metastatic group was made at the left oxter. A tumor piece of 100 mg was fixed under the skin. All animals were sent to the breeding room after becoming conscious.

Specimen collection and pathological examination

Six weeks after implantation, all mice were housed in metabolic cages and maintained in an air conditioned room (24 ± 2℃). They were only allowed free access to water during urine sample collection (8:00 pm that day to 8:00 am the next day). All animal urine was collected in frozen tubes at the sixth week after implantation, and immediately stored at -80℃ until processing. The specimens were collected at the same time. Then all mice were killed, tumors growing on the stomach wall were resected and fixed in 4% formalin, and processed for routine paraffin embedding after careful macroscopic examination. In order to evaluate histologically for liver metastasis or lymph node metastasis or other organ metastasis under microscope, four-micron-thick sections were stained with hematoxylin and eosin, then observed by a blinded pathologist.

Sample pretreatment and derivatization

Each urinary specimen was transferred to a glass cen-

trifuge tube, subsequently centrifuged at 18000 × *g* for 3 min and 50 μL of the supernatant was collected from each sample into a 1-mL EP tube, respectively. The collected supernatant was evaporated to dryness at 60℃ for 24 h, using a vacuum dryer. Then 100 μL THF was added to each of the dried urine extracts and vortex-mixed for 2 min, and 50 μL BSTFA was added to the mixture and vortex-mixed for 2 min. The mixture was incubated at 60℃ and derivatized for 30 min. After returning to the ambient temperature, samples were prepared for GC/MS analysis.

GC/MS analysis

Each derivatized sample of 1 μL was injected splitless into an Agilent 6980 GC system equipped with an HP5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm), electron impact ionization at 70 eV, and a quadrupole mass spectrometric detector (Agilent Technologies, Palo Alto, CA, USA). The column temperature was initially held at 100℃ for 3 min, 10℃/min up to 220℃, then 10℃/min to 280℃, and remained there for 5 min. The injector temperature was 280℃. Carrier gas flow was helium at a constant flow rate of 1.0 mL/min. The interface temperature and the ion source temperature were set at 200℃. Masses were obtained from 100-600 *m/z*. GC total ion chromatograms (TICs) and fragmentation patterns were acquired using GC/MSD ChemStation Software (Agilent Technologies, Palo Alto, CA, USA). Compound identification was performed by comparing the mass spectrum with a standard mass spectrum in the national institute of standards and technology (NIST) mass spectra library. Peaks with similarity index more than 70% were assigned compound names, while those having less than 70% similarity were listed as unknown metabolites[30]. The chromatograms were subjected to noise reduction prior to peak area integration. Any known artificial peaks, such as peaks due to noise, column bleed and BSTFA derivatization procedure, were excluded from the data set. Integrated peak areas of multiple derivative peaks belonging to the same compound were summed and considered as a single compound. The resulting three dimensional matrix included sample information, peak intensities and peak retention time, and was applied to correlation analysis and pattern recognition.

Data processing and pattern recognition

The relative peak area of each compound would be calculated as the response after the peak areas of compounds were integrated. Each sample was represented by a GC/MS TIC. *t* test was employed for statistical analysis. Data were expressed as mean ± SD. The differentially expressed compounds with $P < 0.05$ were considered statistically significant. PCA was used to differentiate the samples and performed using the SPSS 16.0 for Windows.

RESULTS

General state of mice and pathological results

The mean weight of mice was 23.81 ± 0.16 g, 23.87 ± 0.19 g and 23.98 ± 0.19 g for normal group, non-metastasis group and metastasis group, respectively ($P > 0.05$).

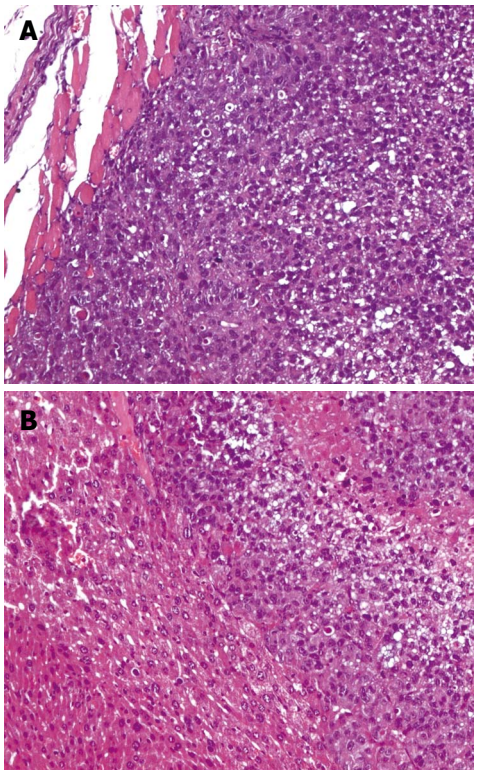

Figure 1 Gastric cancer pathological photographs. A: Gastric cancer cells in mice of the non-metastatic group (HE stain, × 200); B: Gastric cancer metastasis in the liver (HE stain, × 200).

All animals from the three groups were alive at the sixth week. The normal group mice had no tumor and metastasis. The non-metastasis group and metastasis group developed localized tumors at the implanted site, which were poorly-differentiated adenocarcinomas under microscope (Figure 1A). The non-metastasis group tumor tissues (4.28 ± 0.20 g) were located at the left oxter, and have no metastasis in regional lymph nodes, liver and other organs. The metastasis group mice had cancer tissues (4.3 ± 0.3 g *vs* non-metastasis group, $P > 0.05$) in the stomach, while metastatic tumors were also found in liver (Figure 1B), regional lymph nodes, and other organs. Six mice developed metastatic tumors in regional lymph nodes, four in liver, and two in other organs.

Metabolomic profiling of samples

GC/MS TIC chromatograms of urine samples derived from the normal group, the non-metastatic group and the metastatic group are presented in Figure 2. In the GC/MS TICs of urinary samples from the three groups, some peaks were identified based on NIST mass spectra library, and several examples of peaks had statistical significance (Figure 2).

With GC/MS, around 120 signals were detected per sample using mass spectral deconvolution software for peak detection. However, many of them were not consistently found in other samples or were of too low abundance or too poor spectral quality to be obviously assigned to unique metabolites. Several choline, amino acids, and fatty acids could not be found, which may be

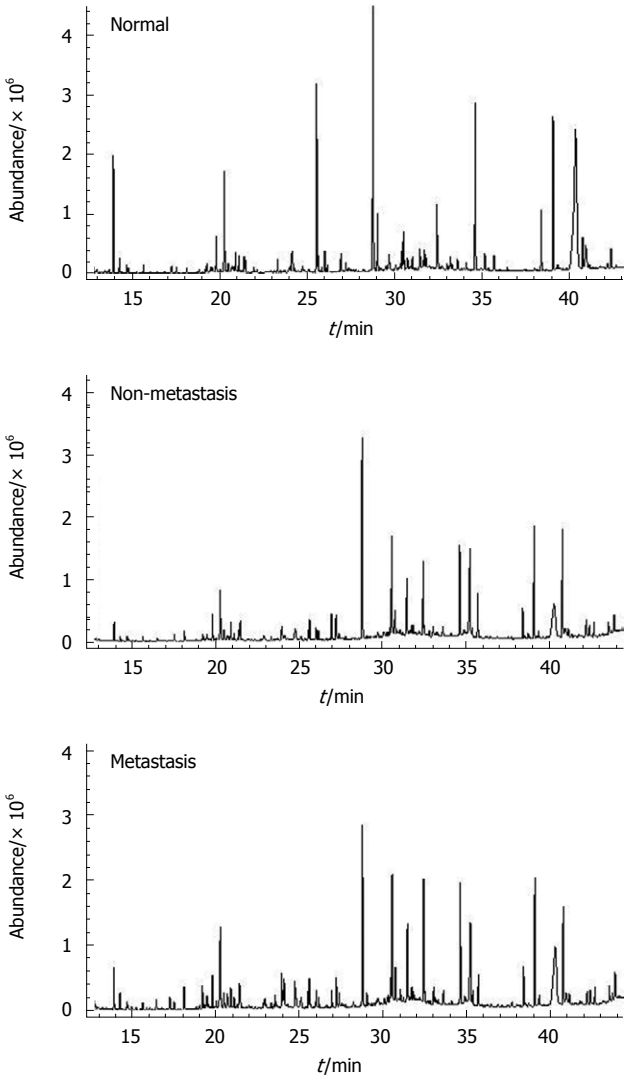

Figure 2 Representative gas chromatography/mass spectrometry total ion chromatograms of the samples from the three groups (normal group, non-metastasis group and metastasis group) after chemical derivatization.

associated with the efficiency of chemical derivatization. Table 1 shows that 46 signals could be auto-identified by the NIST library through comparing with a standard mass chromatogram. The remaining peaks which could not be identified were not listed. In addition, the retention time of metabolites and the match percentage to the NIST library are also listed in Table 1.

Three TIC profiles of consecutively injected samples of the same aliquot are presented in Figure 3, which showed stable retention time with no drift in all of the peaks. The stable TICs reflected the stability of GC/MS analysis and reliability of the metabolomic data.

Urine GC/MS data from the three groups were analyzed. Metabolites selected by *t* test are listed in Tables 2 and 3 after normalization of data. Lactic acid, butanoic acid, propanoic acid, glycerol, pyrimidine, butanedioic acid, malic acid, citric acid, hexadecanoic acid and uric acid were found at higher levels in the urine of cancer group (non-metastasis group and metastasis group) than in normal control group (Table 2). Furthermore, the decreased levels of alanine, butanoic acid, glycerol, L-proline and L-threonic acid were found in the metastasis group as compared with the non-metastasis group. However, the levels of butanedioic acid and myo-inositol were significantly higher in the metastasis group than in the non-metastasis group (Table 3).

Table 1 Urine metabolites of mice in the three groups (normal, non-metastasis and metastasis)

Peak No.	Retention time	Metabolites	Match percent (%)
1	7.196	Lactic acid	91
2	7.508	Acetic acid	90
3	8.105	Alanine	90
4	8.518	Glycine	91
5	8.936	Pentanoic acid, 4-oxo-	94
6	9.412	Butanoic acid	83
7	11.940	Urea	95
8	12.511	Glycerol	91
9	12.590	Silanol	97
10	13.933	Butanedioic acid	97
11	14.293	Propanoic acid	94
12	14.705	Pyrimidine	93
13	14.774	Triacetin	83
14	15.657	2-Piperidinecarboxylic acid	90
15	16.070	L-threonine	87
16	16.460	N-(1-oxobutyl)-Glycine	90
17	17.270	N-(2-methyl-1-oxopropyl)-Glycine	91
18	17.530	(R*,S*)-3,4-Dihydroxybutanoic acid	94
19	19.301	Malic acid	90
20	19.492	N-(3-methyl-1-oxobutyl)-Glycine	98
21	19.566	2,3,4-oxy-Butanal	90
22	19.814	1,2,3,4-oxy-Butane	90
23	20.497	L-proline	96
24	20.909	L-threonic acid	90
25	21.475	Creatinine	96
26	25.108	Hexanedioic acid	90
27	25.632	Arabitol	91
28	25.843	Nonadecane	83
29	26.018	Xylitol	93
30	26.166	Ribitol	91
31	26.838	4-Pyrimidinecarboxylic acid	96
32	26.938	1-Propene-1,2,3-tricarboxylic acid	91
33	27.208	Phosphoric acid	90
34	28.768	Citric acid	91
35	29.032	Myo-inositol	83
36	30.328	Mannonic acid	95
37	30.540	Hydrazone	96
38	30.730	N-Phenylacetyl glycine	93
39	31.037	Silane	91
40	31.449	L-Gluconic acid	99
41	32.422	D-Gluconic acid	91
42	33.025	Dehydrocholic Acid	92
43	34.612	Hexadecanoic acid	99
44	35.691	Uric acid	98
45	38.388	Retinoic acid, methyl ester	95
46	39.065	Octadecanoic acid	99

Peaks in the total ion chromatograms are numbered according to their retention time. The identification of metabolite is based on national institute of standards and technology mass spectra database according to the match of masses (m/z) between the interested peak's fragmentation pattern and that from the standard database.

Pattern recognition and function analysis

A PCA model for gastric cancer was constructed using

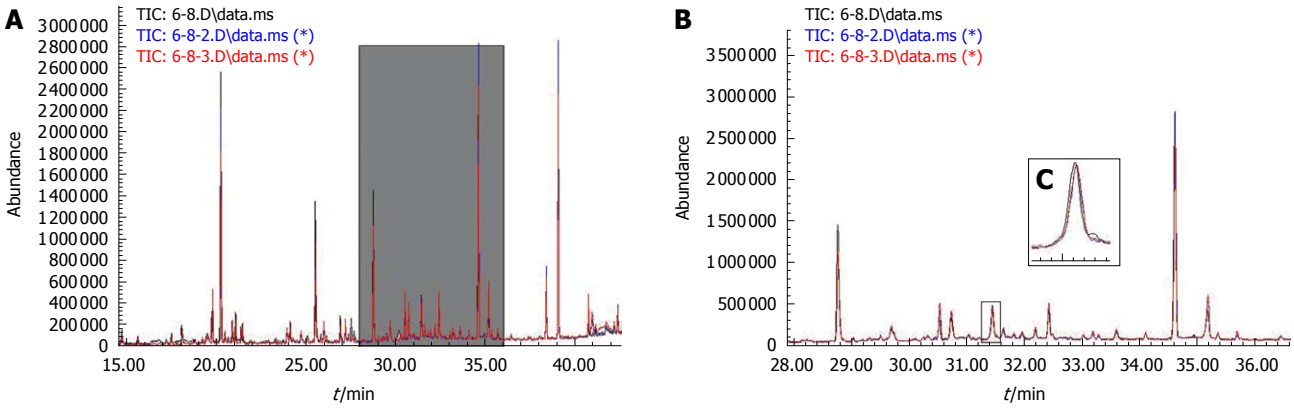

Figure 3 The overlay chromatograms of three parallel samples. A: The total ion chromatograms (TICs) of gas chromatography/mass spectrometry analysis; B: Enlarged part of TIC from 28 to 36 min; C: One peak enlarged.

Table 2 Marker metabolites found in normal and cancer groups

Metabolites	Retention time	*P* value[1]	A (normal)	B (cancer[2])	*R*[3]
Lactic acid	7.196	2.4×10^{-5}	79.24 ± 6.1	187.04 ± 71.99	1.36
Butanoic acid	9.412	0.000	16.79 ± 0.52	27.33 ± 4.98	0.63
Propanoic acid	14.293	0.000	60.58 ± 9.79	147.77 ± 15.3	1.43
Glycerol	12.511	0.000	147 ± 8.98	269.13 ± 50.31	0.83
Pyrimidine	14.705	0.000	61.68 ± 8.05	163.11 ± 12.23	1.64
Butanedioic acid	13.933	0.1×10^{-5}	161.51 ± 5.85	267.89 ± 54.64	0.66
Malic acid	19.301	0.000	10.7 ± 1.91	32.15 ± 1.16	2.00
Citric acid	28.768	1.4×10^{-4}	1291.89 ± 364.74	2164.74 ± 529.58	0.68
Hexadecanoic acid	34.612	4.17×10^{-4}	1347.84 ± 304.67	2066.57 ± 437.28	0.53
Uric acid	35.691	0.000	172.2 ± 17.03	214.52 ± 7.74	0.25

[1]*P* values were calculated based on Student *t* test (significance at $P < 0.05$); [2]Cancer group included the non-metastasis group and the metastasis group; [3]*R* value was calculated from the arithmetic mean values of each group. *R* = (B-A)/A. *R* with a positive value indicates a relatively higher concentration in cancer group while a negative value means a relatively lower concentration as compared with the normal group.

Table 3 Metabolic differences in the two groups

Metabolites	Retention time	*P* value[1]	A (non-metastasis)	B (metastasis)	*R*[2]
Alanine	8.105	0.000	173.75 ± 39.59	19.28 ± 10.63	-0.89
Butanoic acid	9.412	0.000	32.09 ± 1.00	22.58 ± 0.72	-0.30
Glycerol	12.511	0.003	303.23 ± 26.16	235.04 ± 45.64	-0.22
Butanedioic acid	13.933	0.1×10^{-5}	216.36 ± 2.63	319.43 ± 17.89	0.48
L-proline	20.497	0.000	184.99 ± 10.26	117.78 ± 7.05	-0.36
L-threonic acid	20.909	2.28×10^{-4}	284.94 ± 46.47	181.48 ± 37.25	-0.36
Myo-inositol	29.032	0.000	33.08 ± 3.58	114.8 ± 2.20	2.47

[1]*P* values were calculated based on Student *t* test (significance at $P < 0.05$); [2]*R* value was calculated from the arithmetic mean values of each group. *R* = (B-A)/A. *R* with a positive value indicates a relatively higher concentration in metastasis group while a negative value means a relatively lower concentration as compared with the non-metastasis group.

the marker metabolite intensities as variables (lactic acid, butanoic acid, propanoic acid, glycerol, pyrimidine, butanedioic acid, malic acid, citric acid, hexadecanoic acid and uric acid). The PCA scores plot showed that the normal group and cancer group (non-metastasis group and metastasis group) samples were scattered into different regions (Figure 4A). ROC analysis, which was performed using the values determined by the first two components of the PCA model, confirmed the robustness of the PCA model. These first two components could present the majority of all significantly different metabolites among the groups (the percentage is 82.7%). Area under the curve (AUC) value of this PCA model was 1.00 (Figure 4B), which demonstrated a good diagnostic value for gastric cancer. In addition, another PCA model for gastric cancer metastasis constructed by seven marker metabolites (alanine, butanoic acid, glycerol, L-threonic acid, L-proline, butanedioic acid and myo-inositol) could differentiate between the non-metastasis group and the metastasis group (Figure 5A). This PCA model was also validated by

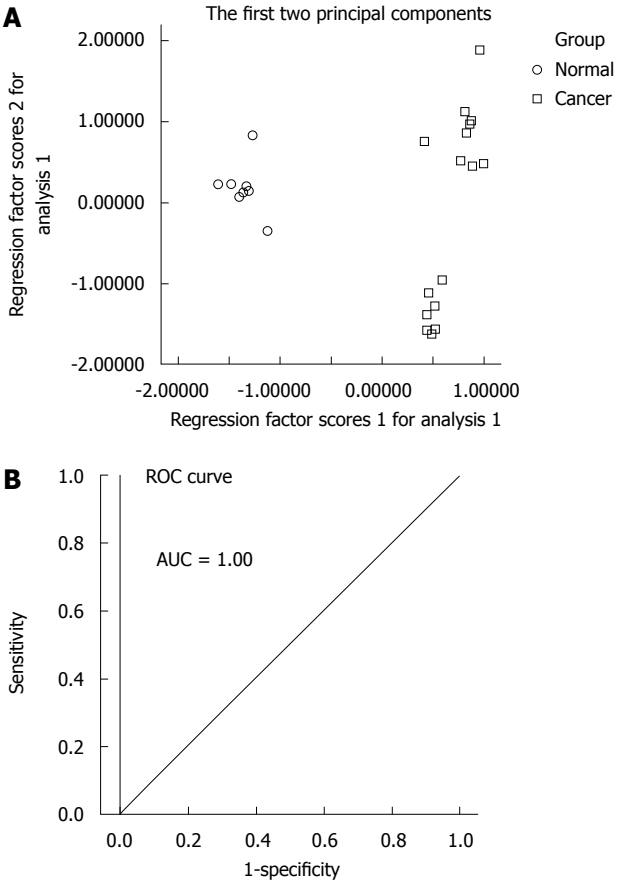

Figure 4 Principal component analysis model and receiver operating characteristic curve for gastric cancer. A: Principal component analysis (PCA) scores plot of gastric tumor specimens from control specimens based on 10 marker metabolites. The PCA scores plot showed different samples (normal group, cancer group including non-metastasis group and metastasis group) were scattered into different regions; B: Receiver operating characteristic (ROC) analysis was performed using the values determined by the first two components. Area under the curve (AUC) = 1.00.

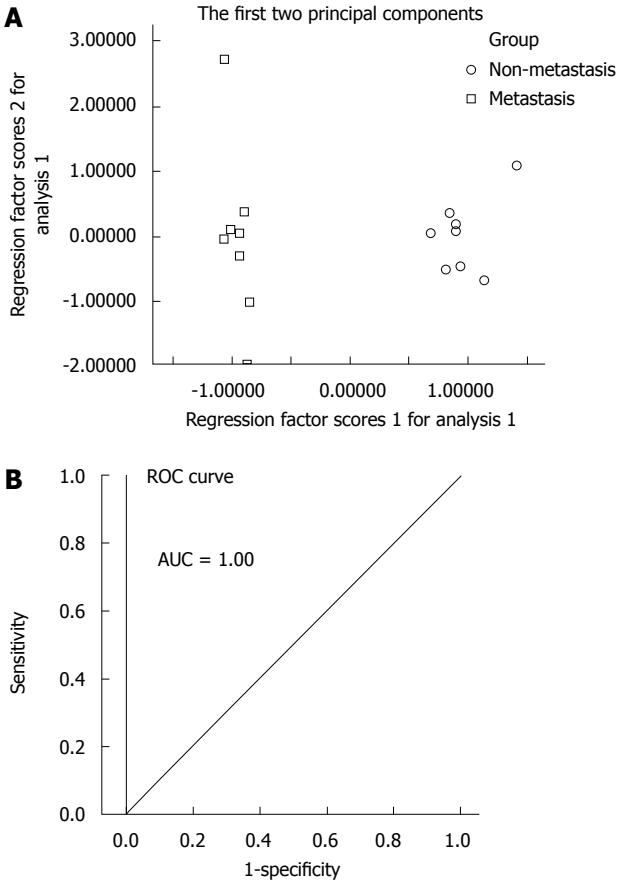

Figure 5 Principal component analysis model and receiver operating characteristic curve for gastric cancer metastasis. A: Principal component analysis (PCA) scores plot of non-metastasis group and metastasis group based on 7 marker metabolites. The PCA scores plot showed the samples from non-metastasis group and metastasis group were scattered into two different regions; B: Receiver operating characteristic (ROC) analysis was performed using the values determined by the first two components. Area under the curve (AUC) = 1.00.

receiver operating characteristic (ROC) analysis (AUC = 1.00, Figure 5B).

DISCUSSION

In this study, we investigated urinary metabolite profiling using GC/MS. This was assessed non-invasively by measuring two voxels (tumor and healthy controls). We have discriminated the gastric cancer model mice from their healthy controls in a PCA analysis of GC-MS urinary metabolite spectra. Moreover, we could also discriminate the gastric cancer metastasis model mice from the non-metastasis model mice by GC-MS and PCA of urinary metabolites. Some marker metabolites were worth investigating in the future. Compared with the normal group, the level of lactic acid was higher in the cancer group urine. It could be explained that glucose is often converted into lactic acid in cancer cells, which is known as the "Warburg effect", and cancer cells have a higher rate of aerobic glycolysis[31]. The levels of butanedioic acid, malic acid and citric acid, intermediates of tricarboxylic acid (TCA) cycle, were also found to be higher in the gastric cancer mice. The abnormalities of these metabolite expressions demonstrated a close correlation of TCA cycle with gastric cancer morbidity along with disordered aerobic respiration and mitochondrial functions. The disorder of aerobic respiration (mainly TCA cycle) and the impairment of mitochondrial enzymes have been reported in other malignancies including colorectal cancer, pheochromocytoma and paraganglioma[22,32,33]. Uric acid, the final metabolite of purines, at enhanced level in cancer mice urine, suggests the abnormalities of purine metabolism in gastric cancer[34]. In our study, the significantly higher levels of glycerol and hexadecanoic acid in cancer than in normal groups were interpreted as increased adipocyte lipolysis in cancer and enhanced expression and function of adipocyte hormone-sensitive lipase (HSL)[35].

Cancer metastasis could be considered as an essential prognostic factor[36]. Figure 5A shows the new constructed tumor metastatic model by seven marker metabolites for the non-metastasis group and the metastasis group. This PCA model was also validated by ROC analysis (AUC = 1.00, Figure 5B). Seven metabolites in this model are capable of predicting the gastric cancer metastasis. Compared with the non-metastasis group, levels of alanine

and glycerol were found to be lower in the metastasis group. Alanine and glycerol could get into the glycolytic pathway through gluconeogenesis, which produced more energy for the tumor progression and metastasis. The decreased level of L-proline in the metastasis group may be interpreted as increased demand for structural proteins synthesis. These proteins, including receptors, membrane channels and enzymes, play an important role in tumor progression and metastasis[37-39]. Moreover, the higher level of myo-inositol in metastasis group urine, was consistent with the reduction of myo-inositol in lung cancer tissues[40]. The amount of myo-inositol may be a potential indicator for gastric cancer metastasis, as it has been reported that the Gly:Myo-inositol ratio may be a useful index for brain tumor classification[41].

What the difference of metabolite changes of butanoic acid and pyrimidine between the normal and the cancer groups, and the decreased levels of butanoic acid and L-threonic acid in the metastasis group indicates remains unclear.

In conclusion, GC/MS revealed detailed information on the metabolic profile of normal and cancer urine and was found to be suitable, in tandem with the PCA model, for the identification of metabolic variations characteristic of the gastric cancer. Furthermore, seven metabolites have been selected, which constructed a diagnostic model for distinguishing the non-metastatic and the metastatic gastric cancer. To our knowledge, this is the first report on urinary metabolomic investigation of gastric cancer metastasis by GC/MS. On the basis of this research, we believe that urinary metabolomic information obtained by GC/MS might play a significant role in the early diagnosis and screening metastasis or recurrence of gastric cancer.

COMMENTS

Background

Gastric cancer is the second leading cause of cancer death worldwide, and in many Asian countries. Tumor metastasis is one of the leading causes of cancer death. Metabolic alterations play a role in the biology of cancer. The urinary metabolites as gastric cancer or tumor recurrence biomarkers can be obtained by investigating the urinary metabolic profiling.

Research frontiers

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and its approaches offer an analysis of metabolite level changes in biological samples. Recently, metabolomic method has shown great potentials in identifying the new diagnostic markers and therapeutic targets for cancers. However, metabolomic studies on cancer metastasis remain scarce.

Innovations and breakthroughs

Recently, metabolomic studies on gastric cancer and colon cancer tissues have been conducted. Compared with tissues and serum, markers acquired from urine are noninvasive and convenient, especially in the patients with recurrent gastric cancer. This is the first report on urinary metabolomic investigation in gastric cancer using gas chromatography/mass spectrometry (GC/MS).

Applications

Potential metabolic biomarkers in urine could be used for early diagnosis and screening the metastasis or the recurrence of gastric cancer.

Terminology

Metabolomics is a post-genomic research field for analysis of low molecular weight compounds in biological systems, and its approaches offer an analysis of metabolite level changes in biological samples. Because of its peak resolution, high sensitivity and reproducibility, GC/MS has been widely utilized in metabolomics.

Peer review

This manuscript evaluates tumor metabolism with a goal to identify possible biomarkers with potential diagnostic value and the potential for prediction of tumor metastasis. The authors concluded that the urinary metabolomic profiling of each group is different, and the selected metabolites might be instructive to clinical diagnosis or screening metastasis for gastric cancer. This is a relevant randomized control trial using an animal model to evaluate a non-invasive method for surveillance of gastric cancer.

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ORIGINAL ARTICLE

Non-alcoholic fatty liver disease: An early mediator predicting metabolic syndrome in obese children?

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Abstract

AIM: To investigate if non-alcoholic fatty liver disease (NAFLD) is an early mediator for prediction of metabolic syndrome, and if liver B-ultrasound can be used for its diagnosis.

METHODS: We classified 861 obese children (6-16 years old) into three subgroups: group 0 (normal liver in ultrasound and normal transaminases); group 1 (fatty liver in ultrasound and normal transaminases); and group 2 (fatty liver in ultrasound and elevated transaminases). We measured the body mass index, waist and hip circumference, blood pressure, fasting blood glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), whole-body insulin sensitivity index (WBISI), lipid profile and transaminases in all the participants. The risk of developing metabolic syndrome (MS) was assessed according to the degree of liver fatty infiltration based on the B-ultrasound examination.

RESULTS: Among the 861 obese children, 587 (68.18%) were classified as having NAFLD, and 221 (25.67%) as having MS. The prevalence of MS in NAFLD children (groups 1 and 2) was 37.64% (221/587), which was much higher than that in non-NAFLD group (group 0, 12.04%) ($P < 0.01$). There were significantly higher incidences concerning every component of MS in group 2 compared with group 0 ($P < 0.05$). The incidence of NAFLD in MS patients was 84.61% (187/221), which was significantly higher than that of hypertension (57.46%, 127/221) and glucose metabolic anomalies (22.62%, 50/221), and almost equal to the prevalence of dyslipidemia (89.14%, 197/221). Based on the B-ultrasound scales, the presence of moderate and severe liver fatty infiltration carried a high risk of hypertension [odds ratio (OR): 2.18, 95% confidence interval (95% CI): 1.27-3.75], dyslipidemia (OR: 7.99, 95% CI: 4.34-14.73), impaired fasting glucose (OR: 3.65, 95% CI: 1.04-12.85), and whole MS (OR: 3.77; 95% CI: 1.90-7.47, $P < 0.01$). The state of insulin resistance (calculated by HOMA-IR and WBISI) deteriorated as the degree of fatty infiltration increased.

CONCLUSION: NAFLD is not only a liver disease, but also an early mediator that reflects metabolic disorder, and liver B-ultrasound can be a useful tool for MS screening.

Key words: Childhood obesity; Non-alcoholic fatty liver disease; Metabolic syndrome; Liver B ultrasonography

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INTRODUCTION

There is a growing concern for non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MS) in obese children[1-3]. NAFLD is a clinicopathological syndrome that ranges from simple steatosis to steatohepatitis, fibrosis or cirrhosis of the liver[4]. It is associated with dyslipidemia, obesity, and insulin resistance, which are the main features of MS. NAFLD and MS often are seen in the same individual[5-8], whereas insulin resistance probably is a key event that links them together. The mortality of patients with NAFLD has increased significantly among the general population, and cardiovascular risk competes with liver-related risk in dictating the final outcome[9,10]. Several prospective studies in adults have demonstrated that NAFLD is associated with an increased incidence of MS and type 2 diabetes mellitus in elderly patients, and independent patients with obesity[11-14]. Thus, NAFLD may be not only a liver disease, but also an early mediator of type 2 diabetes mellitus and MS in adults. However, the impact of NAFLD on MS among the young population is still not clear. The debate remains as to whether NAFLD should be included as one of the components of the MS. This study aimed to assess idiopathic NAFLD among Chinese obese children, to verify the prevalence of MS in NAFLD patients based on B-ultrasound scan, and to investigate whether NAFLD is associated with MS and MS profile, and whether it is an important risk factor for MS as well.

MATERIALS AND METHODS

Study design and population

A total of 861 obese children and adolescents, aged between 6 and 16 years, who were referred to our endocrinology department with the complaint of obesity from January 2004 to September 2009, were enrolled in this study. Based on an accepted criteria for obesity diagnosis in Chinese children[15], all the participants had a body mass index (BMI) that was above the 95th percentile for their age and sex, based on the national reference data in 2004[16]. Exclusion criteria were the known presence of endocrine metabolic or kidney diseases, and the use of medication that altered blood pressure, liver function, and glucose or lipid metabolism. The demographic distribution of subjects is displayed in Table 1. Participants underwent a routine clinical examination, including physical examination, biochemical tests, oral glucose tolerance test (OGTT) and liver ultrasonographic scanning. There was no difference in age and Tanner stages between boys and girls. However, significant differences existed in clinical, anthropometric and laboratory data in relation to sex (Table 1).

Table 1 Characteristics of 861 obese patients

	Girls (n = 263)	Boys (n = 598)
Age (yr)	10.53 ± 2.26	10.81 ± 1.97
Tanner stage (T1/T2-4)	126/137	287/311
BMI (kg/m^2)[a]	27.68 ± 4.24	28.38 ± 3.47
BMI Z-score[a]	4.31 ± 1.58	3.05 ± 1.16
WHR (waist/hip ratio)[a]	0.93 ± 0.07	0.96 ± 0.05
Waist (cm)[a]	86.53 ± 11.83	90.91 ± 10.25
Systolic pressure (mmHg)[a]	114.47 ± 13.50	117.33 ± 12.55
Diastolic pressure (mmHg)	67.85 ± 9.32	68.99 ± 9.00
Cholesterol (mmol/L)	4.48 ± 1.01	4.43 ± 0.93
Triglycerides (mmol/L)	1.79 (0.19-5.17)	1.77 (0.17-10.78)
HDL	1.23 ± 0.28	1.26 ± 0.34
LDL[a]	2.69 ± 0.73	2.51 ± 0.68
AI[a]	2.30 ± 0.83	2.11 ± 0.74
Uric acid (μmol/L)	377.0 (203.2-705.4)	383.0 (198.2-708.5)
Fasting glucose (mmol/L)	4.87 ± 0.58	4.90 ± 0.57
120-min glucose (mmol/L)[a]	6.31 ± 1.52	6.01 ± 1.44
HbA1c (%)	5.74 ± 0.57	5.69 ± 0.52
Fasting insulin (mIU/L)[a]	17.9 (0.3-303.0)	15.3 (0.3-219.0)
HOMA-IR[a]	3.77 (0.07-78.11)	3.33 (0.07-51.59)
WBISI[a]	2.59 (0.39-11.90)	3.37 (0.52-24.19)
ALT (U/L)[a]	37.5 (8.0-283.0)	29.0 (4.0-561.0)

[a]$P < 0.05$ boys *vs* girls. Data are expressed as mean ± SD or median (range). BMI: Body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AI: Atherogenic index; HOMA-IR: Homeostasis model assessment of insulin resistance; WBISI: Whole-body insulin sensitivity index; ALT: Alanine aminotransferase.

The protocol was approved by the Medical Ethics Committee of The Children's Hospital of Zhejiang University School of Medicine. Written informed consent from parents (or guardians) and children (where appropriate) were obtained.

Definition of disease, syndrome and disorders

NAFLD: NAFLD was defined according to the revised definition and treatment guidelines for NAFLD by the Chinese Hepatology Association in February 2006[17], and was diagnosed by means of a protocol using clinical, laboratory and ultrasound examinations in combination. In this study, NAFLD was diagnosed as a diffusely echogenic change on liver B-ultrasonography (fatty infiltration in liver), with or without elevated serum aminotransferase levels and other factors that can cause liver fatty infiltration or aminotransferase elevation, such as hepatitis virus infection, drug-induced injury, and other metabolic diseases, such as Wilson's disease, were excluded. There was no history of current or past alcohol drinking. Two subgroups were classified in NAFLD obese children: group 1 (fatty liver in ultrasound and normal transaminases) and group 2 (fatty liver in ultrasound and elevated transaminases). Group 0 was diagnosed as obese children without liver disorder (normal liver in ultrasound and normal transaminases).

MS: The diagnosis criteria have been described in our previous study[18], which followed the suggestions of the Chinese Diabetes Association and the definition modified from the National Cholesterol Education Program's Adult Treatment Panel Ⅲ (NCEP-ATPⅢ). MS was diagnosed if patients met three or more of the following criteria for age and sex: (1) central obesity; (2) hyperglycemia (fasting glucose ≥ 6.1 mmol/L, or random glucose ≥ 7.8 mmol/L) or impaired glucose tolerance (IGT) or type 2 diabetes; (3) systolic or diastolic blood pressure above the 95th percentile for age and sex; and (4) hypertriglyceridemia (triglyceride concentration > 1.7 mmol/L) or low high-density lipoprotein-cholesterol (HDL-C) (< 1.03 mmol/L). In this study, high blood pressure was based on the percentile data of the 7th edition of Practical Pediatric Text Book by Zhu Fu-Tang[15]. IGT was defined as a glucose level > 7.8 mmol/L but < 11.1 mmol/L at 2 h. Type 2 diabetes was diagnosed according to the criteria of American Diabetes Association in 1997 and World health Organization in 1999.

Laboratory assessment

Height was measured without socks and shoes, and weight was measured in children who were wearing only underclothing. Waist was measured at the midpoint between the lower border of the rib cage and the iliac crest. Hip circumference was determined at the widest circle of the bottom. Pubertal development stages were assessed using Tanner stage criteria. Blood pressure was taken twice using the right arm, with the subject in a quiet sitting position, and the average level was recorded.

Subjects underwent routine biochemical evaluation in the morning before 09:00 h after an overnight fast for at least 8 h. Fasting glucose, insulin, lipids total triglyceride (TG), total cholesterol (TCHO), HDL-C and low-density lipoprotein-cholesterol (LDL-C), liver function [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] and uric acid were detected. Hepatitis serial serological tests (antibodies for hepatitis A-E) and glucose and insulin levels were also recorded during a standard (75 g) OGTT. Blood glucose was determined using a glucose oxidase method (North Biotechnology Company, Beijing, China) with intra-assay and inter-assay coefficient of variation (CVs) of 2.1% and 4.4%. Insulin serum levels were determined by radioimmunoassay (North Biotechnology Company) with intra-assay and inter-assay CVs of 6.4% and 9.7%, respectively. The serum concentrations of TCHO, TG, HDL-C, LDL-C, ALT, AST and uric acid were measured by routine enzymatic methods in our clinical laboratory.

The homeostasis model assessment of insulin resistance (HOMA-IR), based on serum fasting glucose and insulin levels, was used to measure insulin resistance. The whole body insulin sensitive index (WBISI) and the ratio of early insulin increment to early glucose increment (I30-0/G30-0) following oral glucose loading (75 g) were also obtained. HOMA-IR = [FIN (mU/L) × FBG (mg/dL)]/22.5; WBISI = 10000/[FIN (mU/L) × FBG (mg/dL) × average insulin (mU/L) × average glucose (mg/dL)]$^{1/2}$; BMI = weight (kg)/[height (m)]^2 (FIN: Free insulin; FBG: Free blood glucose).

Liver ultrasound examination

Liver ultrasound examination was carried out by one specialist who was unaware of the aims of the study and blinded to laboratory values on the same equipment (GE, LOGIC 500), using a convex 3.5-5.0 MHz probe. Sagittal hepatic sections that encompassed longitudinal images of the right liver lobe and the ipsilateral kidney were obtained. Liver-kidney contrast with two other well-known ultrasonographical findings of fatty liver, vascular blurring and deep attenuation, enabled us to grade fatty change semi-quantitatively. Fatty infiltration was graded semi-quantitatively into four classes[19,20]: no steatosis (class 0), mild steatosis (class 1), moderate steatosis (class 2) and severe steatosis (class 3) (Figure 1).

Statistical analysis

Data were collected using an MS-Excel spreadsheet. Data were analyzed using the JMP Statistical Discovery Software version 7.0 (SAS Institute, Cary, NC, USA). Group comparisons for continuous data were performed using *t* tests for independent means or one-way analysis of variance. A non-parametric test was used to evaluate the significance of abnormally distributed data. For categorical data, we employed the χ^2 test, Fisher's exact test, or binomial test of proportions. Multivariate logistic regression models were used to adjust for covariate effects on the odds ratio (OR). Statistical significance was set at $P < 0.05$.

RESULTS

Prevalence of NAFLD and MS in Chinese obese children

Of 861 obese children, 587 (68.18%) were diagnosed as having NAFLD, and 221 (25.67%) as having MS. The prevalences of hypertension, dyslipidemia, hyperuricacidemia, impaired fasting glucose, IGT and diabetes were 37.28% (321), 42.04% (362), 28.69% (247), 6.38%(55), 8.01% (69) and 1.39% (12), respectively. Moreover, the incidence of NAFLD in MS patients reached 84.61% (187/221), which was significantly higher than that of hypertension (57.46%, 127/221) and glucose metabolic anomalies (22.62%, 50/221), but almost equal to the prevalence of dyslipidemia (89.14%, 197/221).

NAFLD is associated with MS and its components among obese children

To investigate whether liver disorders are associated with MS, participants were divided into three subgroups: group 0 (normal liver in ultrasound and normal transaminases); group 1 (fatty liver in ultrasound and normal transaminases); and group 2 (fatty liver ultrasound and elevated transaminases). Table 2 indicates that sex, age and Tanner stage were comparable among the three subgroups. Nevertheless, the occurrence rates of MS increased with deterioration of liver from 12.04% in group 0 to 29.36% in group 1, and 39.74% in group 2 ($P < 0.05$). The prevalence of hypertension, dyslipidemia, IGT and diabetes in group 2 was significantly higher than that in group 0 ($P < 0.05$). Further investigation indicated that the prevalence of dyslipidemia and IGT increased steadily from group 0 to group 1 and group 2

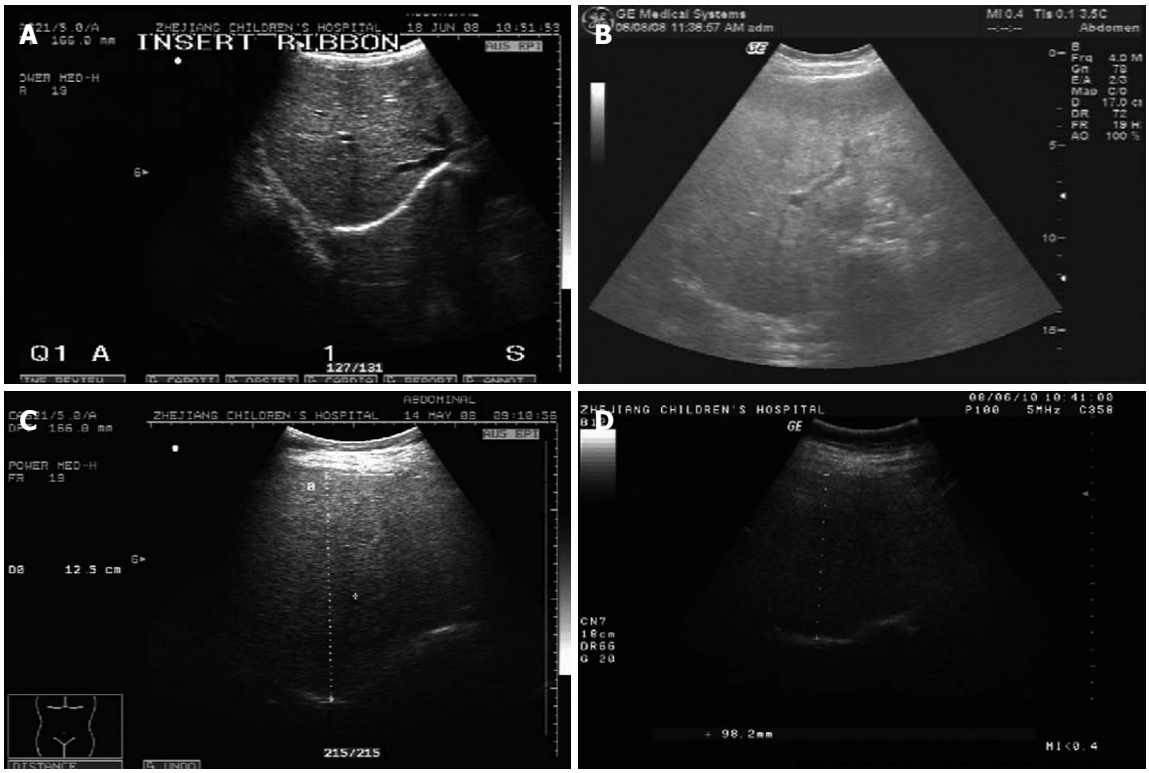

Figure 1 Liver B-ultrasound scans show the panel of four different classes of liver steatosis. A: Class 0: not observed; B: Class 1: mild, liver-kidney contrast without vascular blurring and deep attenuation; C: Class 2: moderate, liver-kidney contrast with vascular blurring, but no deep attenuation; D: Class 3: severe, combination of liver-kidney contrast with vascular blurring and deep attenuation.

Table 2 Comparison of prevalence of metabolic syndrome and its components among three groups

	Group 0 (n = 274)	NAFLD	
		Group 1 (n = 436)	Group 2 (n = 151)
Age (yr)	10.36 ± 2.15	10.59 ± 1.85	10.40 ± 2.28
Sex (M/F)	187/87	305/131	106/45
Tanner stage (T1/T2–4)	130/144	209/227	74/77
BMI	26.88 ± 3.19	28.78 ± 3.96[a]	28.85 ± 3.69[a]
Hypertension	68 (24.82)	180 (41.28)[a]	73 (48.34)[a]
Dyslipidemia	42 (15.32)	204 (46.78)[a]	116 (76.82)[a,c]
Impaired fasting glucose	5 (1.82)	30 (6.88)	20 (13.24)[a,c]
IGT	13 (4.74)	38 (8.72)	18 (11.92)[a]
Diabetes	0 (0.00)	5 (1.14)	7 (4.63)[a,c]
MS	33 (12.04)	128 (29.36)[a]	60 (39.74)[a,c]

[a]$P < 0.05$ *vs* group 0; [c]$P < 0.05$, group 1 *vs* group 2. Data are expressed as percentage, mean ± SD. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; IGT: Impaired glucose tolerance; MS: Metabolic syndrome.

(15.32% to 46.78% to 76.82%; 1.82% to 6.88% to 13.24%, $P < 0.05$). In addition, the levels of triglycerides, cholesterol, ALT and uric acid in group 2 were significantly higher than those in group 1 and group 0 ($P < 0.05$) (Table 3). It was indicated that the NAFLD was closely associated with progression of MS and its components in these obese children.

NALFD is accompanied by insulin resistance in obese children

Insulin resistance is a key event that causes MS in both adults and children[21]. To investigate whether NAFLD is associated with insulin resistance, we performed OGTT and insulin releasing test in all the participants. As for the results of blood glucose, fasting blood glucose and 120-min OGTT glucose levels in group 2 were significantly higher than those in group 0 ($P < 0.05$). The blood level of insulin, including fasting insulin, 30-min OGTT insulin and 120-min OGTT insulin, were significantly increased in groups 1 and 2 as compared with those in group 0 ($P < 0.05$). The HOMA-IR and WBISI reflected insulin resistance and sensitivity respectively. Our analysis indicated that HOMA-IR was elevated significantly in groups 1 and 2, whereas WBISI decreased significantly as compared with that of group 0 ($P < 0.05$) (Table 3). This finding revealed that NAFLD was significantly associated with insulin resistance among these obese children.

MS is associated with liver steatosis found by ultrasound examination

Fatty infiltration is another indicator that reflects liver damage, which can be easily detected by ultrasound examination. Based on B-ultrasound examination, 861 obese children were classified into class 0 (274 cases without steatosis), class 1 (105 cases with mild steatosis), and classes 2-3 (482 cases with moderate and severe steatosis). It was indicated that the relative risk of MS increased to 3.10 [95% confidence interval (95% CI): 1.20-8.00] in class 1 and 3.77 (95% CI: 1.90-7.47) in classes 2-3 ($P < 0.01$) (Table 4). Based on the B-ultrasound scales, the presence of moderate and severe liver fatty infiltration carried a high risk of

Table 3 Clinical and laboratory data of non-alcoholic fatty liver disease compared with obese children without liver disorder

	Group 0 (n = 274)	NAFLD	
		Group 1 (n = 436)	Group 2 (n = 151)
Age (yr)	10.36 ± 2.15	10.59 ± 1.85	10.40 ± 2.28
BMI (kg/m^2)	26.58 ± 3.19	28.85 ± 3.69[a]	28.78 ± 3.96[a]
BMI Z-score	3.17 ± 1.26	3.33 ± 1.37	3.93 ± 1.60[a,c]
Waist circumference (cm)	85.63 ± 10.17	91.58 ± 11.24[a]	92.2 ± 9.31[a]
Father BMI (kg/m^2)	25.12 ± 3.68	25.68 ± 5.78	25.55 ± 3.75
Mother BMI (kg/m^2)	22.83 ± 3.14	23.46 ± 3.61[a]	23.64 ± 3.38[a]
Systolic blood pressure (mmHg)	113.62 ± 11.89	118.30 ± 13.37[a]	118.03 ± 12.73[a]
Diastolic blood pressure (mmHg)	67.80 ± 9.02	69.00 ± 9.38	69.43 ± 8.67[a]
ALT (U/L)	24 (8-74)	29 (4-75)[a]	51.5 (76-561)[a,c]
Uric acid (μmol/L)	352.9 (186.5-603.20)	365.9 (168.10-708.50)[a]	412.5 (7.38-771.30)[a,c]
Triglycerides (mmol/L)	1.4 (0.19-6.76)	1.42 (0.26-5.90)	1.77 (0.28-8.64)[a,c]
Cholesterol (mmol/L)	4.37 ± 0.99	4.34 ± 0.87	4.71 ± 0.99[a,c]
HDL	1.29 ± 0.39	1.23 ± 0.28	1.22 ± 0.28
LDL	2.52 ± 0.66	2.54 ± 0.68	2.68 ± 0.78
AI	2.07 ± 0.73	2.15 ± 0.70	2.31 ± 0.92[a]
Fasting blood glucose (mmol/L)	4.82 ± 0.52	4.88 ± 0.69	4.95 ± 0.55[a]
30-min OGTT glucose (mmol/L)	7.54 ± 1.17	7.73 ± 1.24	7.69 ± 1.21
120-min OGTT glucose (mmol/L)	5.66 ± 1.36	6.31 ± 1.42[a]	6.22 ± 1.63[a]
HbA1c (%)	5.70 ± 0.50	5.71 ± 0.53	5.65 ± 0.62
Fasting insulin (mIU/L)	12.7 (0.3-187.0)	17.0 (0.3-219.0)[a]	18.2 (2.1-303.0)[a]
30-min OGTT insulin (mIU/L)	85.3 (1.8-400.0)	112.0 (0.5-400.0)[a]	106.2 (1.6-400.0)[a]
120-min OGTT insulin (mIU/L)	36.3 (3.2-400.0)	63.6 (1.7-386.0)[a]	69.8 (7.0-400.0)[a]
HOMA-IR	2.61 (0.07-41.56)	3.71 (0.08-51.59)[a]	3.95 (0.48-78.11)[a]
WBISI	4.0 (0.6-16.4)	2.8 (0.9-24.2)[a]	2.6 (0.4-16.7)[a]

[a]P < 0.05 *vs* group 0; [c]P < 0.05, group 1 *vs* group 2. Data are expressed as mean ± SD or median (range). NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AI: Atherogenic index; OGTT: Oral glucose tolerance test; HOMA-IR: Homeostasis model assessment of insulin resistance; WBISI: Whole-body insulin sensitivity index.

developing hypertension (OR: 2.18, 95% CI: 1.27-3.75), dyslipidemia (OR: 7.99, 95% CI: 4.34-14.73), impaired fasting glucose (OR: 3.65, 95% CI: 1.04-12.85), and hyperuricacidemia (OR: 3.76, 95% CI: 2.03-6.96) (Table 4, Figures 2-4). We also determined whether HOMA-IR and WBISI were associated with the degree of fatty liver. It was revealed that the HOMA-IR increased significantly from 2.64 in class 0 to 3.57 in classes 2-3 , but the WBISI decreased from 3.97 in class 0 to 2.67 in classes 2-3 among the obese subjects. All these findings indicated that the scale of fatty infiltration in liver was closely related to MS and insulin resistance among Chinese obese children.

Table 4 Metabolic syndrome in obese patients diagnosed by B-ultrasound

	Scale (0) (n = 274)	Scale (1) (n = 105)	Scale (2-3) (n = 482)	P
Hypertension				
No	206	54	280	
Yes	68	51	202	< 0.05
OR (95% CI)	1.0	2.87 (1.29-6.37)	2.18 (1.27-3.75)	
Dyslipidemia				
No	232	57	210	
Yes	42	48	272	< 0.01
OR (95% CI)	1.0	5.21 (2.23-12.18)	7.99 (4.34-14.73)	
Impaired fasting glucose				
No	269	102	435	
Yes	5	3	47	< 0.05
OR (95% CI)	1.0	1.0 (0.1-9.94)	3.65 (1.04-12.85)	
Impaired glucose tolerance				
No	261	99	432	
Yes	13	6	50	> 0.05
OR (95% CI)	1.0	1.53 (0.27-8.74)	2.9 (0.95-8.87)	
Hyperuricacidemia				
No	232	81	301	
Yes	42	24	181	< 0.01
OR (95% CI)	1.0	1.83 (0.71-4.74)	3.76 (2.03-6.96)	
MS				
No	241	75	324	
Yes	33	30	158	< 0.01
OR (95% CI)	1.0	3.10 (1.20-8.00)	3.77 (1.90-7.47)	

MS: Metabolic syndrome; OR: Odds ratio; CI: Confidence interval.

DISCUSSION

The prevalence of both NAFLD and MS was higher in this study at 68.18% and 25.67%, respectively, than that of 9.6% and 4.2% in the general pediatric population[22]. NAFLD is regarded as an increasing clinical problem in children and adolescents, and accounts for the vast majority of cases with elevated serum liver enzymes[23]. Moreover, NAFLD is known to be related to the factors that predict the development of coronary heart disease, such as dyslipidemia, central obesity and MS.

Apart from that, NAFLD has been shown increasingly and more convincingly to be an important component of MS. In this study, we demonstrated that the prevalence of MS was three times higher in NAFLD obese children than in those without liver disorders (39.74% *vs* 12.04%, P < 0.05). The incidence of each component of MS was also significantly higher in NAFLD subjects (P < 0.05). Moreover, the presence of moderate and severe liver fatty infil-

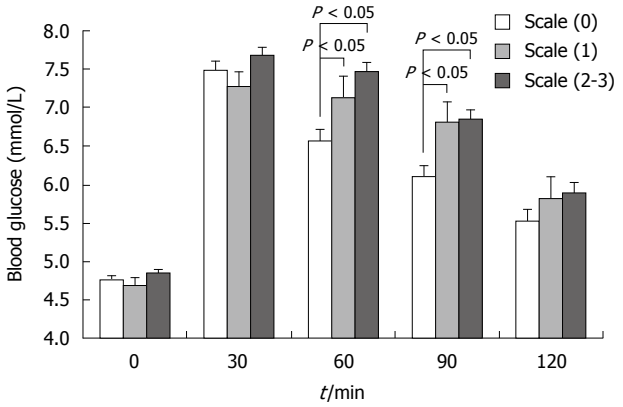

Figure 2 Oral glucose tolerance test in obese patients based on liver B-ultrasound gradings. When patients were stratified according to the presence of liver fatty infiltration based on the B-ultrasound scans, their glucose levels at 60 min and 90 min after tolerance were significantly higher in classes 1-3 ($P < 0.05$) than in class 0, but there was no difference between class 1 and classes 2-3 ($P > 0.05$).

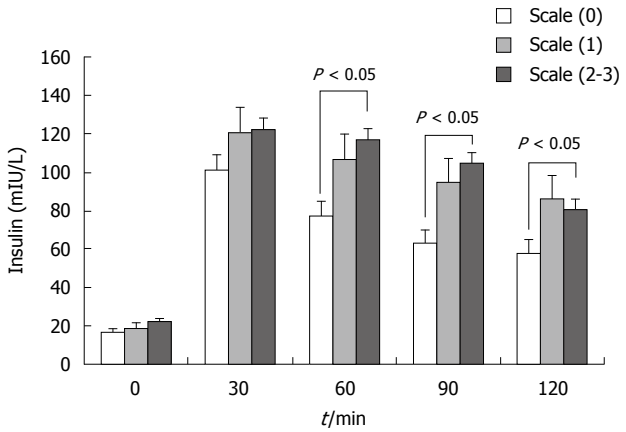

Figure 3 Insulin releasing test in obese patients based on liver B-ultrasound gradings. There was no difference in fasting insulin among the three groups, but 60, 90 and 120 min insulin levels were markedly increased in the class 2-3 groups compared with class 0 ($P < 0.05$).

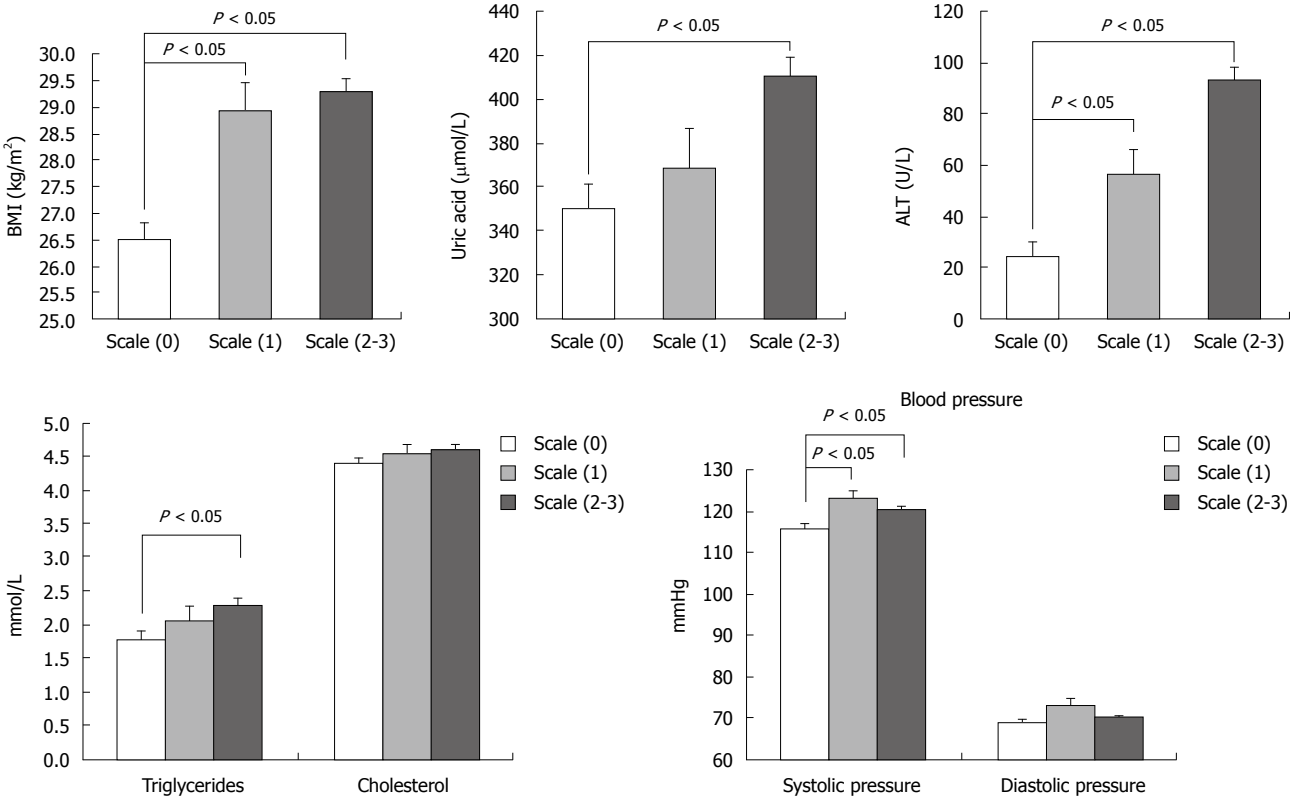

Figure 4 Clinical features of the metabolic syndrome in obese patients based on liver B-ultrasound gradings. Concerning the individual metabolic component, body mass index (BMI), alanine aminotransferase (ALT) and systolic pressure were significantly higher in class 1 and classes 2-3 compared with class 0 ($P < 0.05$), but there was no difference between class 1 and classes 2-3 ($P > 0.05$). The levels of uric acid and triglyceride were only markedly increased in classes 2-3 compared with class 0 ($P < 0.05$). There was no difference in cholesterol levels among the three groups ($P > 0.05$).

tration confirmed by B-ultrasonography carried a high risk of development of MS, which highlights that NAFLD is closely associated with features of MS. In contrast, the incidence of NAFLD in MS patients reached 84.61%, which was significantly higher than that of hypertension (57.46%) and glucose metabolic anomalies (22.62%), and almost equal to the prevalence of dyslipidemia (89.14%). This indicates that NAFLD might be an early stage mediator for prediction of MS.

To date, the biological mechanisms that are involved in the higher risk of developing metabolic disorders in patients with NAFLD are not fully understood. Insulin resistance seems to be the key event. Nevertheless, most obese patients with NAFLD had hyperinsulinemia and higher insulin resistance compared with those without liver disorders, as calculated by fasting insulin, 30 min and 120 min insulin after glucose loading, HOMA-IR and WBISI. In contrast, patients with NAFLD were more

obese and exhibited higher insulin resistance and more marked metabolic complications than those with simple obesity. Fatty liver itself is an insulin resistance status[24], and because hepatic fat accumulation can lead to hepatic insulin resistance, the latter may occur before any alternation in peripheral insulin action or peripheral insulin resistance. Moreover, insulin resistance may cause abnormalities of lipid storage and lipolysis in insulin-sensitive tissues, which may induce increased fatty acid flux from adipose tissue to the liver and result in steatosis[25]. Insulin resistance may also cause lipid peroxidation, which in turn, activates inflammatory cytokines and promotes the progression of innocent steatosis to non-alcoholic steatohepatitis and liver fibrosis[26]. The impairment in fat and glucose metabolism when insulin resistance occurs, can lead to similar biochemical and clinical abnormalities in patients with NAFLD, and sooner or later it will inevitably develop to systemic MS.

An accurate fatty liver diagnosis and staging of non-alcoholic steatohepatitis requires liver biopsy. However, liver biopsy is not performed often in patients, especially in children, with no significant or trivial liver diseases. Most of the patients with liver steatosis can be well-managed without a need for liver biopsy. In our study, steatosis was assessed by liver ultrasonography with a sensitivity of 83% and a specificity of 100% in comparison with the gold standard of histological diagnosis. Therefore, liver ultrasonography is being strongly suggested as a non-invasive study of NAFLD[18,27,28]. It was particularly interesting to find that both the prevalence of MS and every component of MS increased as the liver ultrasonographic grade deteriorated. Moreover, liver fat is highly significantly associated with all components of MS. Compared with abdominal and overall obesity, fatty liver has the highest frequency of clustering, greater specificity, higher positive predictive value and the most attributable risk for detecting risk factors of cardiovascular disease, type 2 diabetes and MS[29,30]. Thus, fatty liver seems to be an early mediator for prediction of other metabolic disorders. Since B ultrasound scan can be easily and widely applied in hospitals, this simple and effective scanning technique may provide a new method of MS screening in the future.

All findings in our study have stimulated interest in the possible role of NAFLD in the development of metabolic complications. Their coexistence in the same individual increases the likelihood of having more severe dysmetabolic status. Several studies in adults also have demonstrated that NAFLD is the primary hepatic complication of obesity and insulin resistance, and may be considered the early hepatic manifestation of MS. Early treatment, such as lifestyle or diet modification, aerobic exercise, or medication (metformin or vitamin E), for NAFLD may not only improve the prognosis of liver disease, but also reduce the occurrence of underlying metabolic and vascular complications. Furthermore, it is also implied that earlier adjustment to mobilizing fat out of the liver might reduce the risks of MS.

ACKNOWLEDGMENTS

We are grateful to Dr. Li-Qin Chen and Zhong-Sheng Yu for their assay expertise. We sincerely thank the parents and children for participating in these studies. We thank the nursing staff of our department for their dedicated care of these young patients during the collection and evaluation of blood samples.

COMMENTS

Background

There is a growing concern for non-alcoholic fatty liver disease (NAFLD) and metabolic syndrome (MS) in obese children. NAFLD and MS often are seen in the same individual, and insulin resistance is a probable key event that links them together. NAFLD may not only be a liver disease, but also an early mediator of type 2 diabetes mellitus and MS in adults. However, the impact of NAFLD on MS among the young population is still not clear.

Research frontiers

NAFLD is now considered a metabolic pathway to advanced liver disease, cirrhosis and hepatocellular carcinoma. Type 2 diabetes mellitus, obesity and dyslipidemia are the principal factors associated with NAFLD, which is now considered the hepatic expression of MS. The risk of liver disease associated with the classical features of MS in children is still unclear. We still need to clarify the mechanisms that are responsible for liver disease progression from pure fatty liver to steatohepatitis and to cirrhosis, and the reasons why only a few NAFLD cases progress to terminal liver failure while others (the majority) will have a cardiovascular outcome.

Innovations and breakthroughs

An accurate fatty liver diagnosis and staging of non-alcoholic steatohepatitis require liver biopsy. However, liver biopsy is not performed often in patients, especially children, with no significant, or trivial liver diseases. Most of the patients with liver steatosis can be well-managed without a need for liver biopsy. In this study, steatosis was assessed by liver ultrasonography, which showed that both the prevalence of MS and every component of MS increased as the liver ultrasonographic grade deteriorated. Liver fat was highly significantly associated with all components of MS.

Applications

Since B ultrasound scan can be easily and widely applied in hospitals, this simple and effective scanning technique may provide a new method of MS screening in the future in the general population.

Peer review

The authors addressed an important subject and described a population of patients referred for obesity with respect to the presence of sonographic evidence for NAFLD, and their metabolic profiles with respect to the insulin axis.

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BRIEF ARTICLE

Perinatal and early life risk factors for inflammatory bowel disease

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Abstract

AIM: To investigate associations between perinatal risk factors and subsequent inflammatory bowel disease (IBD) in children and young adults.

METHODS: Record linked abstracts of birth registrations, maternity, day case and inpatient admissions in a defined population of southern England. Investigation of 20 perinatal factors relating to the maternity or the birth: maternal age, Crohn's disease (CD) or ulcerative colitis (UC) in the mother, maternal social class, marital status, smoking in pregnancy, ABO blood group and rhesus status, pre-eclampsia, parity, the infant's presentation at birth, caesarean delivery, forceps delivery, sex, number of babies delivered, gestational age, birthweight, head circumference, breastfeeding and Apgar scores at one and five minutes.

RESULTS: Maternity records were present for 180 children who subsequently developed IBD. Univariate analysis showed increased risks of CD among children of mothers with CD (P = 0.011, based on two cases of CD in both mother and child) and children of mothers who smoked during pregnancy. Multivariate analysis confirmed increased risks of CD among children of mothers who smoked (odds ratio = 2.04, 95% CI = 1.06-3.92) and for older mothers aged 35+ years (4.81, 2.32-9.98). Multivariate analysis showed that there were no significant associations between CD and 17 other perinatal risk factors investigated. It also showed that, for UC, there were no significant associations with the perinatal factors studied.

CONCLUSION: This study shows an association between CD in mother and child; and elevated risks of CD in children of older mothers and of mothers who smoked.

Key words: Crohn's disease; Ulcerative colitis; Perinatal risk factors; Record linkage

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INTRODUCTION

Both Crohn's disease (CD) and ulcerative colitis (UC) are considered to be immune-mediated disorders, although the exact pathogenetic mechanisms are not yet clear. It is thought that a combination of environmental factors in genetically susceptible people lead to disordered im-

munity and chronic inflammation. Over the last 50 years, there have been large increases in the incidence of CD and UC in the UK, in other western countries[1-3], and more recent increases in Asia[4], which indicate changes over time in the environmental factors that can lead to inflammatory bowel disease (IBD).

In recent decades, there have been changes in the management of births, including large increases in caesarean deliveries, advances in neonatal medicine and substantial reductions in neonatal mortality. As perinatal risk factors have been associated with some immune-mediated diseases including asthma[5-7] and type 1 diabetes[8-10], perinatal risk factors and early life events may be relevant to other immune-mediated diseases, including IBD. One case-control study identified that infectious and non-infectious perinatal health events were linked with 40% of all cases of IBD in the study group[11]. A systematic review and meta analysis of 17 (mainly case-control) studies found a small but significant protective effect of breastfeeding against both CD and UC[12]. However, its authors commented that this finding was far from conclusive, and advocated the need for larger studies. There have been relatively few studies of other perinatal risk factors and IBD.

The aim of this study was to investigate associations between 20 perinatal risk factors and the subsequent development of IBD in children and young adults in a large geographically defined population of South East England. These perinatal risk factors include nine maternal characteristics, such as maternal age, parity, smoking during pregnancy, ABO blood group and social class, and 11 neonatal characteristics, including birthweight, gestational age, head circumference, breastfeeding and Apgar scores.

MATERIALS AND METHODS

Ethical approval for analysis of the record linkage study data was obtained from the Central and South Bristol Multi-Centre Research Ethics Committee (04/Q2006/176).

We used the Oxford record linkage study (ORLS). The ORLS comprises abstracts of records of birth registrations, maternities, day cases and inpatient admissions in a defined geographical region of South East England around Oxford. The maternity data covered all National Health Service (NHS) hospitals in two health districts of the ORLS over the 20-year period from 1970 to 1989. The maternity data are linked to data on all inpatient and day case care for all clinical specialties in the ORLS up to 1999, in two health districts of the ORLS from 1970 to 1999 (population 0.9 million) and a further four adjacent districts (total population 1.9 million) from 1975 to 1999. We used the record linked data to identify cases of subsequent IBD in the children covered by the maternity data. We also used the linked data to identify records of IBD in the mothers, before and after childbirth. The original data comprising the ORLS were abstracted from hospital records by staff who were specifically trained for this purpose by senior clinicians.

We excluded maternity records in the ORLS for 985 abortions, 1560 stillbirths and 1567 early deaths that occurred within 30 d of birth. We also excluded 289 births in which the birthweight was recorded as < 1000 g, because most of these records had implausibly low values and/or substantial missing data for many of the perinatal risk factors that we were investigating. None of these excluded babies were subsequently identified as having IBD. After applying these exclusion criteria, a total of 248 659 births remained in the study.

Cases of CD and UC among offspring and mothers were identified using the following ICD codes on inpatient or day case records: 563.0 and 563.1 for CD and UC, respectively (in the ICD-8th revision), 555 and 556 (ICD-9) and K50 and K51 (ICD-10), when recorded in any diagnostic position on the hospital record. There were 114 and 66 children with both a maternity record and a subsequent admission for CD or UC, respectively. We compared 20 perinatal factors studied in these cases with those in the other 248 479 children without a record of inpatient admission or day case care for CD or UC. Parity was defined as the pregnant woman's number of previous live and still births, as recorded on the ORLS maternity record. The length of "follow-up" for offspring ranged from 30 years for those born in 1970 to 10 years for those born in 1989, with an average follow-up of 18 years.

Statistical methods used included the chi-square test with Yates' correction, odds ratios (ORs) and their 95% CI, and multivariate logistic regression. Statistical significance was accepted at the conventional 5% level. When using logistic regression, all perinatal risk factors that were significant ($P < 0.05$) in the univariate analysis were included in an initial model. Each of the factors that were not significant in the univariate analysis were then re-entered, one at a time, into the regression model. This approach was taken to test whether any perinatal factor that was not significant in the initial univariate analysis, became significant when assessed simultaneously with other significant factors in the multivariate analysis. Cases with missing data for the perinatal risk factors were excluded only for those risk terms that were included in the logistic regression model. Year of birth was routinely included in all of the models, as a potential confounder, because of the different periods of follow-up after different years of birth.

RESULTS

For the 114 and 66 children identified with CD and UC respectively, the age at first admission (mean ± SD) was 17.5 ± 5.1 years and 17.7 ± 6.0 years. Approximately half of the cases of both CD and UC had a first recorded admission in early adulthood (when aged 18 to 30 years) rather than in childhood (Table 1). A majority of cases were female; 59 (52%) for CD and 37 (56%) for UC.

CD

Considering risk factors relating to the maternity, in univariate analysis there was a significant association (P =

Table 1 Age at first day case or inpatient admission for offspring with inflammatory bowel disease *n* (%)

	Age at first hospitalisation (yr) with inflammatory bowel disease						Total
	< 1	1-4	5-9	10-14	15-19	20-29	
Crohn's disease							
Male	1 (2)	1 (2)	4 (7)	11 (20)	18 (33)	20 (36)	55 (100)
Female	0	0	0	11 (19)	19 (32)	29 (49)	59 (100)
Total	1 (1)	1 (1)	4 (4)	22 (19)	37 (32)	49 (43)	114 (100)
Ulcerative colitis							
Male	0	1 (3)	2 (7)	2 (7)	7 (24)	17 (59)	29 (100)
Female	0	1 (3)	3 (8)	6 (16)	11 (30)	16 (43)	37 (100)
Total	0	2 (3)	5 (8)	8 (12)	18 (27)	33 (50)	66 (100)

Table 2 Associations between maternal characteristics and inflammatory bowel disease in the child

Maternal characteristics	No. of births	Crohn's disease			Ulcerative colitis		
		No. of cases	Percent	*P*-value[1]	No. of cases	Percent	*P*-value[2]
Maternal age (yr)				0.19			0.90
14-24	86 544	41	0.047%		21	0.024%	
25-34	142 939	59	0.041%		40	0.028%	
35-49	18 852	14	0.074%		5	0.027%	
Maternal Crohn's disease or ulcerative colitis				0.011[a]			1.00
No	248 132	112	0.045%		66	0.027%	
Yes	530	2	0.380%		0		
Maternal social class				0.10			0.12
Ⅰ & Ⅱ	68 244	21	0.031%		12	0.018%	
Ⅲ	86 869	54	0.062%		32	0.037%	
Ⅳ & Ⅴ	35 510	22	0.062%		11	0.031%	
Marital status				0.081			0.042[a]
Married	224 261	109	0.049%		65	0.029%	
Not married	23 939	5	0.021%		1	0.004%	
Maternal smoking during pregnancy				0.054			0.73
No	110 961	27	0.024%		18	0.016%	
Yes	34 245	16	0.047%		4	0.012%	
Maternal ABO blood group				0.84			0.81
A	101 010	44	0.044%		26	0.026%	
O	105 391	49	0.046%		30	0.028%	
Maternal rhesus Status				0.96			0.97
Negative	39 805	18	0.045%		10	0.025%	
Positive	196 652	87	0.044%		53	0.027%	
Pre-eclampsia				0.29			1.00
No	224 360	99	0.044%		60	0.027%	
Yes	24 250	15	0.062%		6	0.025%	
Parity				0.23			0.59
O	104 210	41	0.039%		25	0.024%	
1+	144 214	73	0.051%		41	0.028%	

[1,2]*P*-values obtained through χ^2 tests, with Yates continuity corrections; [1]Comparing those with Crohn's disease with those without known inflammatory bowel disease (IBD); [2]Comparing those with ulcerative colitis with those without known IBD. [a]$P < 0.05$.

0.011) between CD in the mother and CD in the child (OR = 8.36, 95% CI = 2.06-33.9), based on two cases of CD in both (Table 2). There was a borderline significant association ($P = 0.05$) for maternal smoking and CD in the child (OR = 1.92, 95% CI = 1.03-3.56). There was no significant association between CD and mother's age in the age groupings that we originally selected (< 25, 25-34 and 35+ years; Table 2), but there was a non-significantly increased risk among children of older mothers aged 35+ years, when compared with mothers aged under 35 years (OR = 1.70; 0.97-2.08). Accordingly, we recategorised mothers' age as < 35 years *vs* 35+ years in the multivariate analysis (see below).

We found no significant associations between CD and birth order or with any of the other six maternal risk factors considered, including marital status, ABO blood group, rhesus status and presentation at delivery (Table 2). We found no significant associations between CD and any of the perinatal risk factors relating to the birth, including birthweight, gestational age, caesarean delivery, forceps, Apgar scores and breastfeeding (Table 3).

Using multivariate analysis to assess the independent

Table 3 Associations between characteristics of the births and inflammatory bowel disease in the child

Characteristics of the births	No. of births	Crohn's disease			Ulcerative colitis		
		No. of cases	Percent	*P*-value[1]	No. of cases	Percent	*P*-value[2]
Presentation at delivery				0.47			1.00
Vertex	158302	52	0.033%		26	0.016%	
Other	8311	1	0.012%		1	0.012%	
Caesarean birth				0.72			0.81
No	223793	104	0.046%		63	0.028%	
Yes	18025	10	0.055%		3	0.017%	
Forceps delivery				1.00			0.95
No	210100	99	0.047%		58	0.028%	
Yes	31718	15	0.047%		8	0.025%	
Sex				0.56			0.28
Male	127829	55	0.043%		29	0.023%	
Female	120823	59	0.049%		37	0.031%	
No. of babies				0.53			0.37
1	243269	113	0.046%		63	0.026%	
2+	5390	1	0.019%		3	0.056%	
Gestational age (wk)				0.72			0.60
24-37	21912	8	0.037%		5	0.023%	
38-41	173868	82	0.047%		51	0.029%	
42-47	20567	12	0.058%		3	0.014%	
Birth weight (g)				0.72			0.46
1000-2999	58553	31	0.053%		14	0.024%	
3000-3499	168149	73	0.043%		43	0.026%	
3500+	21151	10	0.047%		9	0.043%	
Head circumference (cm)				0.34			0.99
< 34	34681	13	0.037%		6	0.017%	
34-35	39128	15	0.038%		6	0.015%	
35-36	38528	6	0.016%		7	0.018%	
36+	51035	14	0.027%		7	0.014%	
Breastfeeding				0.89			0.67
Artificial	50966	17	0.033%		10	0.020%	
Breastfed	117364	36	0.031%		18	0.015%	
Apgar 1 score				0.34			0.99
1-5	21356	14	0.066%		6	0.028%	
6-8	64469	27	0.042%		16	0.025%	
9-10	140267	57	0.041%		37	0.026%	
Apgar 5 score				0.87			0.61
1-5	884	0			1	0.11%	
6-8	4229	2	0.047%		0		
9-10	148835	43	0.029%		23	0.015%	

[1,2]*P*-values obtained through χ^2 tests, with Yates continuity corrections; [1]Comparing those with Crohn's disease with those without known inflammatory bowel disease (IBD); [2]Comparing those with ulcerative colitis with those without known IBD.

significance of perinatal risk factors, there was a significantly increased risk of CD among the offspring of mothers who were aged 35+ years, compared with those aged under 35 years (OR = 4.81, 95% CI = 2.32-9.98), and an increased risk of CD among children of mothers who smoked during pregnancy compared with those who did not (2.04, 1.06-3.92). Numbers were too small to warrant inclusion of maternal CD in the multivariate analysis (Table 4).

UC

For UC, in univariate analysis there was only a (marginal) significantly reduced risk for mothers who were not married ($P = 0.042$), although this was based on only one case of an unmarried mother with a child with UC (Tables 2 and 3). Numbers were too small to warrant inclusion of marital status, i.e. a stratum with just one case, in the multivariate analysis. Using multivariate analysis, there were no other significant associations between UC and any of the other 19 perinatal risk factors relating to either the mother or the birth (Table 4).

DISCUSSION

A strength of our study is that we have investigated 20 perinatal risk factors for IBD, unlike other studies that have mostly investigated one, two or only a few factors. The study is based on a geographically defined population, covering prospective data collected over 30 years. Another important strength is that information about the perinatal risk factors and the main outcome measure - IBD in offspring - were collected independently of each other. They were subsequently brought together independently through systematic record linkage, such that information collected for each risk factor was not influenced by knowledge of

Table 4 Perinatal factors with significant, independent effect on Crohn's disease in the child

Perinatal risk factor	Odds ratio	95% CI
Maternal age (yr)		
14-34	1.00	Ref.
35-49	4.81	2.32-9.98
Maternal smoking during pregnancy		
No	1.00	Ref.
Yes	2.04	1.06-3.92

the outcome measure. Our study is therefore not subject to potential interviewer and recall bias, e.g. about whether the mother smoked during pregnancy, which can affect studies based on interviews or self-reporting, and which provide much of the evidence about IBD and perinatal risk factors. The Oxford record linkage study has also been used as the basis of previous studies of perinatal risk factors[7,10,13,14].

The study has several limitations. There was variable follow-up after birth, with shorter durations of follow-up for those born in the more recent years of the study period. However, there was at least 10 years follow-up for all IBD cases among offspring. Maternities during the early years of the study period, and among younger mothers, had fewer years of pre-pregnancy inclusion to ascertain maternal IBD, while data for three of the 20 risk factors (social class, smoking and breastfeeding), were not available for the first four years of the study period. The study would not have identified offspring who were diagnosed with IBD after migrating out of the ORLS region, which would reduce the number of observed cases of IBD.

The identification of cases of IBD in the offspring was restricted to those who were admitted as inpatients or day cases. We will have missed some cases of IBD where the only inpatient or day case admission was for a diagnostic endoscopy and biopsy in patients with suspected IBD, and where the pathology results were not available to create a record of the diagnosis at the time of discharge. We will also have missed people without any day case or inpatient care. Migration over time in the Oxford region population would also have lowered our observed incidence of IBD, particularly among adults. Our age-specific cumulative incidence rates of 1.6 and 0.9 per 100 000 for CD and UC among 0-29 year olds, and 1.0 and 0.5 respectively among 0-19 year olds, are lower than those in some UK studies, but comparable with those in other UK studies[2,3,15-18].

There were two cases of CD in both the mother and child. Previous studies have identified associations between both maternal CD and maternal UC and IBD in offspring[19,20], which are part of a well-established genetic association of IBD in families[21-23]. There was some evidence of increased risks of CD, but not UC, among children whose mothers smoked during pregnancy. Previous studies have reported no association with smoking during pregnancy[19,24], although one case-control study found modest protection against both CD and UC[25]. Our data refers to smoking by the mother: in the general literature, there is strong evidence that active smoking increases the risk and perhaps the severity of CD[26,27]. Although we did not have information on smoking status of the IBD subjects themselves, it is of some interest that we found an association between maternal smoking and CD.

Some studies have identified slightly increased risks of IBD among children from lower socio-economic groups[11,20], others have reported reduced risks of CD among children from lower socio-economic backgrounds[18,28], and some have found no association between social background and IBD[29,30]. More generally, studies that have investigated the relationship between IBD and socio-economic group have often reported conflicting findings[31-37]. Overall, this indicates that any possible association between socio-economic background and subsequent IBD in children is probably quite weak. We did not find a significant association between social class and either CD or UC.

A systematic review and meta analysis of (mainly case-control) studies reported a small but significant protective effect of breastfeeding against subsequent IBD in offspring[12], although it concluded that new larger studies were required. More recent studies have shown little association between breastfeeding and IBD[19], or even increased risks of CD[30,38]. We found no association between breastfeeding at the time of discharge from hospital and IBD, although this includes those who subsequently discontinued breastfeeding after discharge, and is therefore an incomplete marker of breastfeeding.

Through the use of multivariate analysis, we found higher risks of CD, but not UC, among children of older mothers (aged 35+ years). This is consistent with a Swedish study that reported an increased risk of paediatric CD among female offspring born to older mothers[35], although other studies have identified no link between mother's age and IBD in children[25,39]. Births among older mothers are sometimes associated with increased risks of prenatal medical and obstetric complications, intrapartum complications, perinatal and neonatal morbidity and mortality, as well as increased subsequent risks of various disorders. It is possible that children born to older mothers may be more exposed, or more susceptible, to factors associated with the aetiology of subsequent CD, but not with UC, in their children. It is also possible that our finding on maternal age, though significant, was a chance one, especially as our study investigated 20 perinatal factors. It is worth noting, however, that the finding was highly significant ($P < 0.01$ in multivariate analysis).

We found an indication of reduced risks of IBD, particularly UC, among children of mothers who were unmarried at the time of birth, which is consistent with findings from Sweden[11] and Australia[28].

It has been suggested that caesarean section might increase the risk of subsequent IBD in children, because there is less exposure to maternal bacteria than in vaginal delivery[40]. The reasoning behind this is that, according to the hygiene hypothesis, inadequate exposure to microorganisms in early life might result in higher levels of immune-mediated pathology in later life. Although one study found an increased risk of CD for elective caesarean

sections[28], another found no association for either CD or UC[19]. We also found no association for either CD or UC.

We found no association between maternal parity and IBD. Although increased risks of IBD have been reported occasionally for first born[41,42], or subsequent siblings[43], most studies have found no association between birth order and IBD[11,20,25,30,39]. We also found no association between IBD and any of the other perinatal factors studied, including pre-eclampsia, birthweight, gestational age and Apgar score. These perinatal factors have not usually been associated with IBD in previous studies[11,19,25,28,30].

To summarise, of the 20 perinatal risk factors investigated in this study, we found that maternal CD, smoking during pregnancy and advanced maternal age were associated with increased risks of CD in offspring. For UC, there were no factors associated with increased risks after multivariate adjustment. This, and the fact that the few factors that were associated with CD had quite small effect sizes, suggests that perinatal risk factors have only a minor role in the aetiology of IBD.

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COMMENTS

Background

Both Crohn's disease (CD) and ulcerative colitis (UC) are considered to be immune-mediated disorders, although the exact pathogenetic mechanisms are not yet clear. Perinatal risk factors have been linked with other immune-mediated diseases, including asthma and type 1 diabetes. Other than a suggested, small protective effect of breastfeeding, little has been reported on the role of perinatal factors for either CD or UC.

Research frontiers

This study investigated associations between 20 perinatal risk factors relating to the maternity or the birth and subsequent inflammatory bowel disease (IBD) in offspring in the Oxford region, UK. Risk factors investigated included maternal characteristics such as maternal age, IBD, social class, marital status, smoking in pregnancy, ABO blood group, rhesus status and parity; and characteristics of the birth such as caesarean delivery, number of babies delivered, gestational age, birthweight, breastfeeding and Apgar scores.

Innovations and breakthroughs

The study found increased risks of CD among children of mothers with CD, among children of mothers who smoked during pregnancy, and of older mothers aged 35+ years. There were no significant associations between CD and the 17 other perinatal risk factors investigated, and no associations for UC.

Applications

The findings indicate that these three perinatal risk factors might have some influence on subsequent IBD in children. Overall, however, perinatal factors appear to have a limited role in the aetiology of IBD. This study will help stimulate further research into the influence of perinatal risk factors on IBD. The findings should also provide an important source of information for future systematic reviews and meta analyses of perinatal factors and IBD.

Terminology

Odds ratios were used to assess any increased risks of developing IBD. These denote the chance or odds of developing IBD for a child exposed to a given perinatal risk factor (e.g. caesarean delivery) as a ratio of the chance or odds for a child not exposed to caesarean delivery. The study used record linkage of maternity exposure data and IBD outcome data, which were collected independently of each other.

Peer review

This is a very well written original article. I would like to congratulate the authors on such a nicely done original paper that contributes a lot of new information about perinatal and early risk factors for IBD.

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BRIEF ARTICLE

Probiotic *Lactobacillus rhamnosus* downregulates *FCER1* and *HRH4* expression in human mast cells

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Abstract

AIM: To investigate the effects of four probiotic bacteria and their combination on human mast cell gene expression using microarray analysis.

METHODS: Human peripheral-blood-derived mast cells were stimulated with *Lactobacillus rhamnosus* (*L. rhamnosus*) GG (LGG®), *L. rhamnosus* Lc705 (Lc705), *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) and *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12) and their combination for 3 or 24 h, and were subjected to global microarray analysis using an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array. The gene expression differences between unstimulated and bacteria-stimulated samples were further analyzed with GOrilla Gene Enrichment Analysis and Visualization Tool and MeV Multiexperiment Viewer-tool.

RESULTS: LGG and Lc705 were observed to suppress genes that encoded allergy-related high-affinity IgE receptor subunits α and γ (FCER1A and FCER1G, respectively) and histamine H4 receptor. LGG, Lc705 and the combination of four probiotics had the strongest effect on the expression of genes involved in mast cell immune system regulation, and on several genes that encoded proteins with a pro-inflammatory impact, such as interleukin (IL)-8 and tumour necrosis factor alpha. Also genes that encoded proteins with anti-inflammatory functions, such as IL-10, were upregulated.

CONCLUSION: Certain probiotic bacteria might diminish mast cell allergy-related activation by downregulation of the expression of high-affinity IgE and histamine receptor genes, and by inducing a pro-inflammatory response.

Key words: Probiotic bacteria; Mast cells; Microarray; Allergy; IgE receptor

Peer reviewer: Dr. William R Parker, PhD, Assistant Professor, Department of Surgery, Duke University Medical Center, Box 2605, Durham, NC 27710, United States

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INTRODUCTION

Mast cells are multifunctional regulator cells that are located at strategic host-environment interfaces including skin, vascular barriers and gastrointestinal tract, where they encounter antigens and pathogens, as well as commensal microbes. In the healthy intestinal mucosa, mast cells constitute 2%-3% of the cells of the lamina propria[1]. Mast cells are very heterogeneous cells that are traditionally classified by the content of their specific proteases tryptase and chymase. A mast cell subtype that contains only tryptase (MCT) is predominant in the lung and intestine, whereas mast cells that contain tryptase and chymase (MCTC) are prevalent in the skin and conjunctiva[2]. Mast cell subtypes can even vary between different parts of the same organ. Thus, MCT cells are enriched in the mucosal layer of the intestine but MCTC cells outnumber them in the intestinal submucosa. However, this classification is thought to be interchangeable and can be shaped according to the microenvironment of the cells[3].

Mast cells participate in a variety of physiological functions, such as epithelial secretion and permeability, blood flow, peristalsis, neuroimmune interactions, and wound healing. One significant task for mast cells is host defence against pathogenic microbes. By secreting several mediators including histamine, proteases, lipid mediators and pro- and anti-inflammatory cytokines, mast cells regulate the immune system and interact with other immune cells[4]. The multifunctionality of mast cells can explain why they are also involved in the pathogenesis of many inflammatory diseases, such as allergy. The number of mast cells and the amount of mast-cell-derived mediators, such as histamine, which is the key mediator in allergy, are increased at sites of allergic inflammation. The released mediators induce mucus and electrolyte secretion, smooth muscle contraction, nerve-cell activation and other symptoms common in allergic reactions[5]. The regulation of mast-cell mediators is complex. The best characterized mechanism of mast cell activation is high-affinity IgE receptor (FcεR1)-mediated activation[6]. IgE-receptor aggregation induces multiple signaling pathways that control the secretion of allergy-related mediators, such as histamine and leukotrienes, and the induction of T helper cell 2 (Th2) type cytokine and tumor necrosis factor (TNF) gene transcription[7]. The inflammatory effects of the released histamine are mediated by histamine receptors H1-H4[8]. However, some of the mast cell mediators, including interleukin (IL)-10 and histamine[9] can have anti-inflammatory effects and decrease inflammation[10].

Probiotics are defined as live microbes that have beneficial effects on the host's health when administered in adequate amounts[11]. In clinical intervention studies, certain probiotics have been documented to be effective in the prevention and treatment of various clinical conditions. The most promising results of the health effects of probiotics have been discovered in studies of diarrhea[12], allergy[13], irritable bowel syndrome (IBS)[14,15], and respiratory infections[16,17]. The effects of probiotics are suggested to be strain-specific, although one strain can have multiple influences[18]. In the treatment of a complex and heterogeneous condition such as IBS, the use of combinations of different strains of probiotics can have advantages over using a single strain[14]. The most investigated and used probiotic genera are *Lactobacillus* and *Bifidobacterium*. *Lactobacillus* strains have been effective in beneficially modulating commensal microbes and inhibiting pathogen adhesion to gut mucosa. *Lactobacillus* and *Bifidobacterium* have been shown to produce antimicrobial agents and to alleviate symptoms of allergy[19]. Dairy *Propionibacterium,* which has been observed to exclude pathogenic microbes from gut mucosa[20], has also been used as a probiotic.

Evidence from two clinical trials performed with the combination of four probiotic bacteria, i.e. *Lactobacillus rhamnosus* (*L. rhamnosus*) GG (LGG), *L. rhamnosus* Lc705 (Lc705), *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) and *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12), suggests that the consumption of such combination alleviates the symptoms in IBS patients[14,21]. As mast cells are believed to be important in regulating intestinal immunity and perhaps also intestinal sensory functions, we chose to study the effects of the above probiotic combination and each bacterium alone on the global gene expression of primary peripheral-blood-derived human mast cells.

MATERIALS AND METHODS

Cell culture

Freshly collected buffy coats from healthy adult blood donors were provided by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). The health of the subjects for blood donation is strictly controlled. The donors must be 18-65 years of age, free of infections including HIV, hepatitis B and C, and free of allergic symptoms and most chronic illnesses including autoimmune diseases. Mononuclear cells were purified from heparinized blood by Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Mast cell precursor cells were isolated by positive immunomagnetic selection using indirect CD34 MicroBead Kit and MACS® separation columns (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions. After selection, the $CD34^+$ cells were cultured for 9-11 wk in serum-free Stem Span™ cell culture medium (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin and streptomycin (GIBCO BRL, Grand Island, NY, USA), human recombinant stem cell factor (SCF; Peprotech, Rocky Hill, NJ, USA), IL-3 (Peprotech), IL-9 (Peprotech), IL-6 (Peprotech) and human low-density lipoprotein, as previously described[22]. Cultured mast cells were phenotypically and functionally similar to mature MCTC cells as measured by c-kit and IgE receptor (FcεR1) expression, and the presence of chymase, tryptase, heparin and histamine in their granules, as described previously in detail[23].

Bacterial strains

L. rhamnosus GG (ATCC 53103), *L. rhamnosus* Lc705 (DSM 7061), *P. freudenreichii* ssp. *shermanii* JS (DSM 7067) and *B. animalis* ssp. *lactis* Bb12 (DSM 15954) were provided by Valio Research Centre (Helsinki, Finland). LGG and Lc705 were grown as previously described[24]. PJS was grown under optimized aerobic conditions at 30℃ in whey broth (Valio) twice for 2 d at a concentration of 2%. Bb12 was grown under anaerobic conditions at 37℃ in de Man, Rogosa and Sharpe (MRS) broth enriched with 5 g/L L-cysteine hydrochloride monohydrate (Merck, Darmstadt, Germany) three times for 17-18 h at a concentration of 2%[25]. LGG, Lc705, PJS and Bb12 were grown to logarithmic growth phase and the number of bacteria was determined by counting in a Petroff-Hauser counting chamber. *Chlamydia pneumoniae* isolate Kajaani 6 (Cpn) was used as a reference strain, and was obtained from the National Institute for Health and Welfare (Helsinki, Finland) and was propagated as described previously[22].

Mast-cell stimulation with bacteria

After differentiation, mast cells were collected and resuspended in fresh Stem Span™ medium that contained antibiotics and SCF as described above. Single live bacterial strains were added to the cell culture in a bacterium-to-cell ratio of 5:1 based on preliminary experiments (data not shown). When the stimulation was performed with the combination of bacteria, each of the four strains was dosed in a bacterium-to-cell ratio of 1.25:1, thus the total bacterium-to-cell ratio of the combination was 5:1. The cells were incubated with bacteria for 3 or 24 h at 37℃ in 5% CO_2. After incubation, mast cells were separated from the medium by centrifugation, and the medium was aliquoted and stored at -20℃. The cells were washed free of bacteria, lysed and homogenized in RLT buffer (Qiagen, Valencia, CA, USA) and stored at -70℃ before RNA isolation. All experiments were performed with mast cells obtained from three different blood donors. For analysis, the cells from different donors of each experiment were pooled.

RNA isolation and microarray

Total RNA was isolated from the cell lysates using RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. Microarray experiments were performed at Biomedicum Genomics (Helsinki, Finland) using an Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). Integrity and purity of the RNA were verified with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA was prepared and hybridized using the two-cycle protocol of the GeneChip® Expression Analysis kit (Affymetrix) according to the manufacturer's recommendations. Double-stranded cDNA was synthesized from total RNA. Next, biotin-labeled cRNA was transcribed from the cDNA, and the cRNA was fragmented and hybridized. The hybridization reactions were scanned using a GeneChip Scanner 3000 (Affymetrix).

Microarray analysis

The robust multiarray averaging algorithm[26] in the Bioconductor simpleaffy package[27,28] was used to calculate expression estimates from GeneChip signal intensity data. To provide better precision and accuracy and to overcome interpretation problems related to conflicting id gene references[27], an updated probe set definition was used[29], based on Ensemble gene information. In contrast to the default Affymetrix chip description file with 54675 probe sets, the used custom chip description file (version 11.0.1) contained 17492 unique Ensemble gene probe sets. The significance of differential expression was assessed using the empirical Bayes moderated paired *t* statistics (eBayes function) in the limma package, followed by intensity-based hierarchical Bayes analysis[30,31]. In the analysis, a moderated paired *t* test was computed by constructing cell line effects in the linear model. All *P* values were adjusted for multiple hypotheses testing using the bootstrapped q value approach in the qvalue package[32]. Genes with *P* values $\leqslant 0.05$ were identified as significantly differentially expressed.

The GOrilla Gene Enrichment Analysis and Visualization Tool[33] was used to discover functional categories that were enriched at either end of a gene list sorted by the moderated *t* test score, which was calculated using limma. The input gene set was used as a background. Clustering and visualization of the gene expression differences were done using MeV Multiexperiment Viewer tool and hierarchical clustering with Euclidean as a distance, and average linkage clustering as the linkage method[34,35].

Quantitative reverse transcriptase-polymerase chain reaction

To validate the microarray data, TaqMan® real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described[22] for selected genes. Total RNA from the same samples used for the microarray experiments was reverse transcribed to cDNA using random hexamers (Invitrogen, Paisley, UK) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA, USA) were chosen for detection of *IL8* (Hs00174103_m1), *CCL2* (Hs00234140_m1), *IL10* (Hs00174086_m1), *HRH4* (Hs00222094_m1), *FCER1A* (Hs00758600_m1) and *FCER1G* (Hs00610227_m1). Transcripts for *TNF-α* were detected by using sense primer 5'-GCTGCACTTTGGAGTGATCG-3', antisense primer 5'-GTTTGCTACAACATGGGCTACAG-3' and probe 5'-FAM-CCCAGGCAGTCAGATCATCTTCTC-GA-BHQ1-3'. Samples were analyzed in triplicate. β-Actin was used as an endogenous normalization control. Relative quantification was determined by standard $2^{-\Delta\Delta CT}$ calculations[36].

RESULTS

Gene expression profiling

In order to explore the effects of different probiotic bacteria or their combination on mast cells, transcrip-

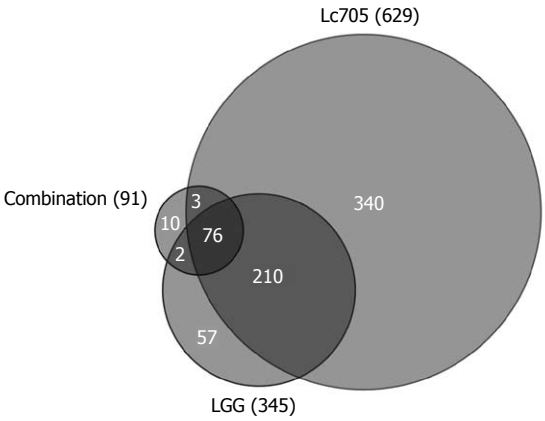

Figure 1 Schematic representation of statistically significant changes ($P < 0.05$) in mast-cell gene expression after 24 h stimulation with *Lactobacillus rhamnosus* Lc705, *Lactobacillus rhamnosus* GG and the combination of four probiotic bacteria. LGG: *Lactobacillus rhamnosus* GG; Lc705: *Lactobacillus rhamnosus* Lc705.

tional changes of the bacteria-stimulated mast cells were studied using a whole genome microarray. A total of 42 Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays were used to analyze gene expression profiles of unstimulated mast cells or those stimulated with live LGG, Lc705, PJS, Bb12, or the combination of these four bacteria. Cpn was included in the analysis as a reference bacterium, which represented a non-probiotic, pathogenic microbe with certain known transcriptional effects on mast cells[22]. Results are representative of three independent experiments, each performed with cells from three donors at two different time points (3 and 24 h). After 3 h bacterial stimulation, the differences in the levels of mast-cell gene expression were so low that no statistical significance was observed as compared to unstimulated samples (data not shown). At 24 h, however, a statistically significant change was observed in 698 genes. Numbers of differentially expressed genes are illustrated in Figure 1. Lc705, LGG and the combination of the four probiotic strains were the most effective stimulators. Bb12 affected the expression of only one gene, and PJS and Cpn failed to change mast-cell gene expression significantly. Lc705 affected mast-cell gene expression the most by changing the expression of 629 genes significantly. Of these genes, 288 were upregulated and 341 were downregulated. LGG altered the expression of 345 genes (160 upregulated and 185 downregulated), and the combination of the four probiotic strains changed the expression of 91 genes (57 upregulated and 34 downregulated). Raw data of the microarray analysis are available at http://ekhidna.biocenter.helsinki.fi/poxo/download_data.

Gene functional category analysis and hierarchical clustering

To characterize the biological significance of the differentially expressed mast-cell genes, a gene ontology category analysis was performed. GOrilla Gene Enrichment Analysis and Visualization Tool[33] was used to discover whether some functional categories showed statistically significant, concordant differences between the stimulated sample and the unstimulated sample. The enrichment analyzes were carried out for the whole array gene set, including the genes that did not reach statistical significance in the array analysis, that were ranked based on their moderated *t* values. For the analysis, the ranked genes were sorted in the order of the highest and lowest *t* value of each sample. The analysis software used the sorted gene lists to classify the genes of each sample into gene ontology categories[37] by their biological function. Selected functional groups and examples of genes that represented each category are depicted in Table 1.

Stimulation of mast cells with LGG, Lc705, Bb12, combination of probiotics, or Cpn resulted in upregulation of genes that belong to categories that involve immune system processes, regulation of programmed cell death, and leukocyte activation. Stimulation with LGG, Lc705, Bb12 or the combination of probiotics suppressed mast-cell genes that are involved in general cell activities and metabolism such as cell cycle and lipid biosynthetic processes. Lc705 was found to suppress genes that are related to mast-cell activation.

Representative genes from the functional categorization (Table 1) were selected to compare the expression patterns of different samples. Mast cells have a central role in many inflammatory responses as well as in allergy, therefore, immunologically relevant genes and genes involved in mast cell activation and mediator release were selected. In order to compare the expression of the selected genes, a hierarchical comparison analysis with the MeV two-way hierarchical clustering method was performed. As expected, LGG and Lc705 but also the combination of four probiotics showed similar expression patterns in the clustering analysis, and were therefore considered to alter mast-cell gene expression in a similar manner (Figure 2). Genes that are involved in similar processes were also grouped: genes that encode Toll-like receptor (TLR) 1, nucleotide-binding oligomerization domain containing 2 (NOD2), FCER1A and FCER1G (high-affinity IgE receptor 1 gene subunits α and γ, respectively) grouped together; and genes that encode proteins with inflammatory functions, such as IL-1β and IL-8 constituted another distinct cluster.

Although there were differences in the intensity of the expression levels, all bacteria except PJS induced upregulation of mast-cell genes included in the functional group of immune system regulation. Examples of upregulated genes in this category were *TLR1, TLR6, NOD2, IL1B, IL8,* chemokine (C-C motif) 2 (*CCL2*), *TNF* and *IL10*. Genes that are involved in regulation of programmed cell death, such as caspases 3 and 8 (*CASP3* and *CASP8*) and cyclin-dependent kinase inhibitor 1B (*CDKN1B*), were observed to be upregulated in LGG, Lc705, and the combination-stimulated cells. LGG, Lc705 and the combination also significantly enhanced the expression of the cluster of differentiation 8 A (*CD8A*) and lymphocyte cytosolic protein 2 (*LPC2*) genes, which are involved in leukocyte activation.

Cyclin A2 (*CCNA2*) and mitogen-activated protein kinase 12 (*MAPK12*) genes involved in the regulation of cell

Table 1 Representative subsets of differentially expressed mast-cell genes after bacterial stimulation classified into functional categories

Go class	Description	*P* value						Gene examples
		LGG	Lc705	PJS	Bb12	Combination	Cpn	
Upregulation								
GO:0002376	Immune system process	3.48E-16	2.95E-13	-	5.25E-7	5.66E-12	1.64E-4	*TNF, IL1B, IL8, IL10, CCL2, TLR1, TLR6, NOD2*
GO:0043067	Regulation of programmed cell death	2.77E-8	9.63E-8	-	7.18E-4	3.49E-6	-	*CASP3, CASP8, CDKN1*
GO:0045321	Leukocyte activation	8.97E-9	2.17E-4	-	-	2.23E-4	-	*CD8A, LCP2*
Downregulation								
GO:0007049	Cell cycle	4.76E-5	9.61E-7	-	-	5.81E-4	-	*CCNA2, MAPK12*
GO:0033033	Regulation of mast cell activation	-	7.51E-4	-	-	-	-	*FCER1A, FCER1G*
GO:0008610	Lipid biosynthetic process	-	-	-	2.02E-5	-	-	*LPGAT1*

The three selected highly upregulated or downregulated functional categories from the enrichment analysis performed with GOrilla Gene Enrichment Analysis and Visualization Tool. *P* value is the enrichment *P* value reported by GOrilla. The resultant categories reflect the gene expression differences of the bacteria-stimulated sample compared to unstimulated sample in 24 h time point. LGG: *Lactobacillus rhamnosus* GG; Lc705: *Lactobacillus rhamnosus* Lc705; PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS; Bb12: *Bifidobacterium animalis* ssp. *lactis* Bb12; Cpn: *Chlamydia pneumoniae* isolate Kajaani 6; TNF: Tumor necrosis factor; IL-1B: Interleukin-1β; IL-8: Interleukin-8; IL-10: Interleukin-10; CCL2: Chemokine (C-C motif) 2; TLR1: Toll-Like receptor 1; TLR6: Toll-Like receptor 6; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; CASP3: Caspase 3; CASP8: Caspase 8; CDKN1: Cyclin-dependent kinase inhibitor 1; CD8A: Cluster of differentiation 8 A (T cell surface glycoprotein); LCP2: Lymphocyte cytosolic protein 2; CCNA2: Cyclin A2; MAPK12: Mitogen-activated protein kinase 12; FCER1A: Fc fragment of IgE high affinity I receptor for α polypeptide; FCER1G: Fc fragment of IgE high affinity I receptor for γ polypeptide; LPGAT1: Lysophosphatidylglycerol acyltransferase 1.

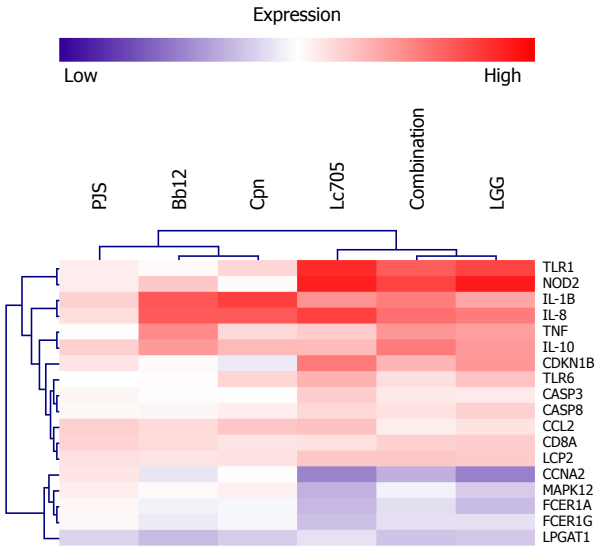

Figure 2 Two-way hierarchical clustering of representative genes selected from the functional category analysis (Table 1). Data are average expression differences between unstimulated and bacteria-stimulated mast cells from three independent experiments at the 24-h time point. Analysis was performed using MeV software. PJS: *Propionibacterium freudenreichii* ssp. *shermanii* JS; Bb12: *Bifidobacterium animalis* ssp. *lactis* Bb12; Cpn: *Chlamydia pneumoniae* isolate Kajaani 6; Lc705: *Lactobacillus rhamnosus* Lc705; LGG: *Lactobacillus rhamnosus* GG; TLR1: Toll-Like receptor 1; NOD2: Nucleotide-binding oligomerization domain-containing protein 2; IL-1B: Interleukin-1β; IL-8: Interleukin-8; TNF: Tumor necrosis factor; IL-10: Interleukin-10; CDKN1B: Cyclin-dependent kinase inhibitor 1B; TLR6: Toll-Like receptor 6; CASP3: Caspase 3; CASP8: Caspase 8; CCL2: Chemokine (C-C motif) 2; CD8A: Cluster of differentiation 8 A (T cell surface glycoprotein); LCP2: Lymphocyte cytosolic protein 2; CCNA2: Cyclin A2; MAPK12: Mitogen-activated protein kinase 12; FCER1A: Fc fragment of IgE high affinity I receptor for α polypeptide; FCER1G: Fc fragment of IgE high affinity I receptor for γ polypeptide; LPGAT1: Lysophosphatidylglycerol acyltransferase 1.

cycle, and the latter also in IgE receptor signaling, were observed to be downregulated in cells stimulated with LGG, Lc705, and the combination of four probiotics. Stimulation of mast cells with Lc705 significantly suppressed the expression of *FCER1A* and *FCER1G* genes. The same trend was also observed after stimulation with LGG and the combination, although these changes in the analysis failed to reach statistical significance. Bb12 was found to suppress the expression of *LPGAT1* (lysophosphatidylglycerol acyltransferase 1), a mast cell gene that is related to lipid biosynthesis, and also the other bacteria studied had a similar impact on the expression of this gene.

Manual screening of the array data

To gain further insight into the probiotic-induced changes in mast-cell activation, the array data set was screened for additional genes that were related to the IgE receptor signaling pathway and mast-cell activation and immunomodulation. The screening was performed manually using the list of genes that reached statistical difference in the array analysis at the 24-h time point (http://ekhidna.biocenter.helsinki.fi/poxo/download_data). The gene that encodes phospholipase C (PLC) that is involved in the mast-cell IgE receptor signaling pathway was observed to be significantly downregulated by LGG and Lc705. The gene that encodes prostaglandin E2 receptor (*PTGER*), which mediates the effects of this inflammatory prostaglandin, was also downregulated by LGG and Lc705. Although only Lc705 was categorized in the functional group as downregulating mast-cell activation, LGG-stimulated cells also reached statistical significance in suppressing *FCER1A* expression in the microarray analysis. Both Lc705 and LGG also suppressed the expression of the gene that encodes mast-cell histamine H4 receptor (HRH4). Expression alterations of the genes that encode the end-products of the IgE receptor signaling pathway were also screened, but no changes were observed in the mast-cell expression of leukotrienes, heparin or Th2 type cytokines, such as IL-3, IL-4, IL-5 and IL-15 after bacterial stimulation.

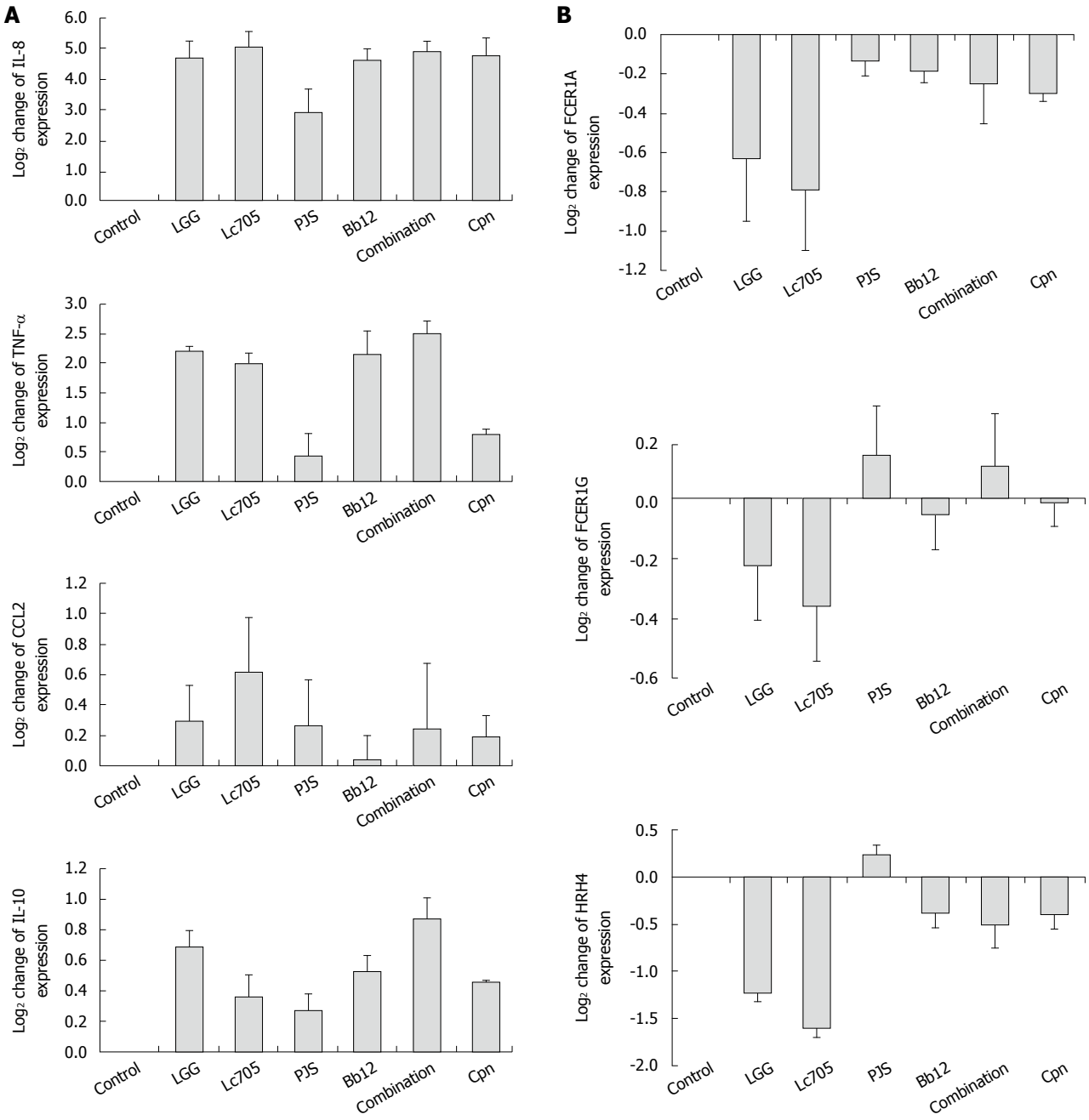

Figure 3 Verification of mast-cell microarray results by quantitative reverse transcriptase-polymerase chain reaction with seven selected genes that are involved in mast-cell immune system regulation (A) and mast-cell activation (B). Gene expression was quantified after 24 h stimulation with four probiotic bacteria; *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus rhamnosus* Lc705 (Lc705), *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS), *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12) and their combination or with *Chlamydia pneumoniae* isolate Kajaani 6 (Cpn). Data are mean values ± SE of three independent experiments. IL: Interleukin; TNF-α: Tumor necrosis factor-α; CCL2: Chemokine (C-C motif) 2; FCER1A: Fc fragment of IgE high affinity I receptor for α polypeptide; FCER1G: Fc fragment of IgE high affinity I receptor for γ polypeptide; HRH4: Histamine H4 receptor.

Quantitative RT-PCR

Findings from the microarray analysis were verified by quantitative RT-PCR. Seven genes with statistical significance after 24 h stimulation with at least one of the bacteria in the array analysis or in the functional categorization analysis were selected. Four of the genes encoded proteins that are involved in mast-cell regulation of immunological events: *IL8, TNF-α, CCL2* and *IL10* (Figure 3A). Three of the genes encoded proteins that are involved in mast-cell activation and in allergy: *FCER1A, FCER1G* and *HRH4* (Figure 3B). With minor differences in the levels of intensities, all of the selected genes followed a similar expression pattern detected in the microarray analysis, and thus confirmed the results of the microarray data.

DISCUSSION

In the present study, microarray analysis was used to gain a broad understanding of interactions between probiotic bacteria and human primary mast cells. We report here, that live probiotic bacteria have species-specific effects on human mast cells. The most significant changes in mast-

cell gene expression were observed in the regulation of genes related to mast-cell activation and mediator release, including *FCER1A, FCER1G* and *HRH4*, and immunological responses such as *IL8, TNF, CCL2* and *IL10*.

In the microarray analysis, lactobacilli affected mast-cell gene expression more than the other bacteria. Stimulation of mast cells with both LGG and Lc705 significantly downregulated the expression of the high-affinity IgE receptor subtype α (*FCER1A*) and HRH4 (*HRH4*) genes after 24 h stimulation. In addition, Lc705 stimulation downregulated the gene expression of FCεR1 receptor subtype γ (*FCER1G*). PJS, Bb12, the combination, or Cpn did not have an effect on *FCER1* and *HRH4* genes. FCεR1 plays a key role in mediating the allergy-related IgE-dependent activation and degranulation of mast cells[38] as demonstrated in FCεR1-deficient mice that fail to show allergic reactions after sensitization[39]. After FCεR1 aggregation, mast cells release inflammatory mediators, such as histamine. Histamine is the key mediator in causing the symptoms of allergy, and it also has a potent role as a modulator of immune responses[40]. The effects of histamine are mediated through histamine receptors H1-H4 that are expressed on the surface on many cell types, including mast cells and other inflammatory cells as well as epithelial cells[41]. The most recently discovered HRH4 has been shown to have mainly immunomodulatory effects[41]. The expression of histamine receptors is suggested to be influenced by inflammatory stimuli[40]. In addition, modification of histamine receptor gene expression is suggested to play a role in the pathogenesis of allergy, atherosclerosis and rheumatoid arthritis[7]. By suppressing the expression of *FCER1* and *HRH4* genes, probiotic lactobacilli could attenuate mast-cell activation and release of allergy-related mediators.

To obtain more evidence of the possible ability of probiotics to suppress mast-cell activation, the array gene set was manually screened for other genes involved in IgE receptor signaling. Expression of the gene that encodes PLC, which is involved in the release of intracellular calcium and in mast-cell degranulation[39], was suppressed significantly after Lc705 and LGG stimulation. Additionally, the gene that encodes expression of the member of the mitogen-activated protein kinase (MAPK) family, *MAPK12*, that participates in the signaling events that lead to the production of Th2 type cytokines IL-3, IL-4, IL-5 and IL-13, and in the generation of eicosanoids[38], was also downregulated in Lc705 and LGG-stimulated mast cells. These findings suggest that Lc705 and LGG have an inhibitory effect on mast-cell genes that are involved in the IgE signaling cascade, beyond inhibition of IgE receptor gene expression.

Our results with human primary mast cells are in line with those of two previous studies of the effects of non-pathogenic bacteria on human or mouse mast cells. *Escherichia coli* (*E. coli*) K12 strain has been found to downregulate *FCER1A* in the malignant human mast cell line (LAD3)[42]. The other study has reported that *E. coli* strain DSM 17252 inhibits mast-cell degranulation in mouse peritoneal mast cells[43]. Commensal non-pathogenic *E. coli* and probiotic LGG and Lc705 seem to downregulate mast-cell IgE responses similarly. These results suggest that commensal and probiotic bacteria do not stimulate mast cells but rather diminish their activation. However, it is worth noticing that this effect is not universal response to bacteria, because not all probiotic bacteria or pathogenic Cpn affected the gene expression of high-affinity IgE receptor similarly.

Probiotic bacteria were also observed to alter the expression of genes that have inflammatory functions. In the functional categorization analysis, the expression of a category of immune system process that contains, for example, a gene that encodes an inflammatory mediator that is also regulated by the MAPK pathway, TNFα[44], was upregulated in mast cells stimulated with LGG, Lc705, Bb12 and the combination. The categorization analysis also highlighted upregulated functions in known inflammatory genes such as *IL8, CCL2,* and *IL1B* in cells stimulated with LGG, Lc705, Bb12 and the combination. The same genes were also upregulated in mast cells stimulated with Cpn, which is in line with our previous study in which Cpn elicited pro-inflammatory effects in mast cells[22]. However, no upregulation in any of the Th2 type cytokine genes in mast cells after probiotic stimulation in the gene microarray was observed. In the prevention of the symptoms in allergic diseases, probiotics have been suggested to elicit low-grade inflammation and thus shift the immune response away from the allergy-related Th2 type inflammation[45,46]. In *in vitro* studies, probiotic bacteria have been observed to induce the secretion and expression of Th1 type cytokines in monocytes, macrophages and dendritic cells[47-49]. Additionally, in a clinical study, low-grade inflammation induced by LGG in allergy-prone children has been proposed to be one mechanism to prevent atopic diseases[45]. The ability of LGG, Lc705, Bb12 and the combination to induce in mast cells pro-inflammatory rather than Th2 type cytokine expression could have contributed to the observed beneficial low-grade inflammatory response in the above-cited study. Our findings in mast cells support the idea that some probiotic strains shift the mast-cell-mediated immunological response from the Th2 to Th1 type, by inducing expression of pro-inflammatory mediators.

Mast cells stimulated with LGG, Lc705, Bb12, the combination, or with Cpn also induced the expression of a gene that encodes anti-inflammatory IL-10. Mast-cell-derived IL-10 has been observed to be crucial in restriction of chronic skin inflammation in hypersensitivity reactions[50]. Probiotic *Bifidobacterium* strains have been observed to stimulate IL-10 production in immune cells[51]. IL-10 induction in mast cells could participate in balancing the inflammatory impact. *In vivo*, the combination of affected genes after bacterial stimulation is likely to be more important than a change in the expression of any single gene. Thus, the downregulation of *FCER1* and *HRH4* genes combined with the upregulation of *IL10* after stimulation with probiotic *Lactobacillus* might be involved in downregulation of inflammatory responses in allergy and other inflammatory diseases in which mast cells are known to a play role, such as atherosclerosis, rheumatoid

arthritis, inflammatory bowel disease and IBS[5,52-54]. As Cpn upregulated only *IL10* without affecting *FCER1* and *HRH4,* mast cells stimulated with pathogenic Cpn might not have the same clinical effects as those stimulated with probiotic bacteria.

In addition to FCεR1 and HRH4, the function of mast cells is regulated through a variety of other receptors, such as TLRs. In our previous study, we have shown that TLR2 is a receptor for LGG, which triggers the nuclear factor-κB signaling cascade, which leads to expression of different cytokines in human primary macrophages[55]. In addition, probiotic bacteria have been shown to suppress mast-cell degranulation by interrupting FCεR1-mediated signaling through TLR2[56]. The paradox of the ability of probiotic LGG and Lc705 to diminish mast-cell activation, but enhance the mast-cell immune response, could be regulated through the same receptor, TLR2.

PJS failed to change mast-cell gene expression significantly at either time point. Previously, PJS has been observed to induce TNFα expression after 3 h stimulation, and IL-10 expression after 3 and 24 h stimulation in human peripheral blood mononuclear cells (PBMCs)[24]. It could be that mast cells are unresponsive to PJS unlike the PBMC population. It could also be that the quantitative spectrum of microarray is a limiting factor, especially if the expression differences between unstimulated and stimulated samples are low or if the gene is expressed in low quantities[57]. In our experiments, the differences in the gene expression levels of the microarrays were relatively low, which is in accordance with other microarray studies performed with probiotic bacteria[58].

Crosstalk between probiotic bacteria and intestinal mast cells is likely to occur mainly through gut epithelial cells[59]. Probiotic bacteria or their products could incidentally translocate to the lamina propria through intestinal M cells and make direct contact with immunological cells such as mast cells. Additionally, mast cells are suggested to have the possibility to be in direct contact with bacteria or bacterial fragments in atherosclerotic plaques[53,60]. Fragments of Firmicutes and Proteobacteria, which are probably of intestinal origin, as well as Cpn have been detected from atherosclerotic plaques[60]. The intestinal barrier is believed to leak also in healthy humans, which would allow probiotic bacteria to access spaces that contain macrophages, dendritic cells and mast cells. Even though *in vivo* evidence of direct contact between mast cells and probiotic bacteria in the human intestine is missing, *in vitro* interaction studies performed with direct contact between bacteria and host cells, such as mast cells, are required for better understanding of the molecular basis of the immunomodulative properties of probiotic bacteria.

The mast cells differentiated by the peripheral-blood-derived isolation method comprise mainly the MCTC phenotype[23], whereas the gut contains both MCTC and MCT phenotypes; the latter being the predominant mucosal mast-cell phenotype[2]. However, the MCT and MCTC phenotypes both express high-affinity IgE receptors and produce a variety of inflammatory mediators. The responses observed in this study could be further evaluated in a more physiological context, e.g. in *ex vivo* culture models using mast cells derived from human intestinal tissues, or in *in vivo* animal or clinical studies.

The present study is believed to be the first to describe the effects of probiotic bacteria on human mast cells. Our data suggest that especially probiotic *L. rhamnosus* Lc705 and *L. rhamnosus* GG could diminish mast-cell activation and the effects of allergy-related mediators by down-regulating expression of the high-affinity IgE and HRH4 receptors, and by stimulating mast-cell immune responses. Mast cells are important mediators of allergic responses on host surfaces including the intestine, therefore, we propose that mast cells participate in regulating the beneficial immunological responses to probiotic bacteria.

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COMMENTS

Background

Probiotic bacteria are widely used to prevent or relieve symptoms of various clinical conditions, such as intestinal disorders and allergy. However, it is not fully understood what make probiotics effective.

Research frontiers

Mast cells are important immunological cells that have many functions. Mast cells also participate in the pathogenesis of many inflammatory diseases, with allergy being the best-known example. In the prevention of the symptoms in allergic diseases, probiotics have been suggested to elicit low-grade inflammation. In the present study, the authors explored for the first time the role of human mast cells in contributing to the beneficial effects of probiotic bacteria.

Innovations and breakthroughs

In the present study, the authors found that probiotic lactobacilli strains *Lactobacillus rhamnosus* (*L. rhamnosus*) *GG* and *L. rhamnosus* Lc705, but not propionibacteria or bifidobacteria, downregulated expression of high-affinity IgE and histamine H4 receptors, and enhanced mast-cell immune activity, and thus, might diminish the impact of the allergenic response.

Applications

Understanding the mechanisms by which probiotic bacteria elicit their health effects is crucial when designing and using different probiotic strains for specific preventive or therapeutic purposes.

Terminology

Probiotic bacteria are defined as live microorganisms that have beneficial effects on human health. High-affinity IgE receptor mediates mast-cell activation and histamine production in allergy. Histamine H4 receptor is a protein on mast cells that mediates the effects of histamine.

Peer review

In this well-designed study, the authors explored the action of four probiotic strains and their combination on human mast-cell global gene expression. The analysis revealed changes in genes involved in immune responses and mast-cell activation, which are especially involved in the pathogenesis of inflammatory diseases, such as allergy. The authors suggest that lactobacilli are able to diminish the impact of allergenic responses by downregulating expression of high-affinity IgE and histamine H4 receptors, and by enhancing mast-cell immune activity. The study addresses an interesting and important question.

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BRIEF ARTICLE

N-Acetyltransferase 2 genetic polymorphisms and risk of colorectal cancer

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Abstract

AIM: To investigate the possible association between meat intake, cigarette smoking and N-acetyltransferase 2 (NAT2) genetic polymorphisms on colorectal cancer (CRC) risk.

METHODS: Patients with CRC were matched for gender and age to healthy controls. Meat intake and cigarette smoking were assessed using a specific frequency questionnaire. DNA was extracted from peripheral blood and the genotypes of the polymorphism were assessed by polymerase chain reaction-restriction fragment length polymorphism. Five NAT2 alleles were studied (WT, M1, M2, M3 and M4) using specific digestion enzymes.

RESULTS: A total of 147 patients with colorectal cancer (76 women and 90 men with colon cancer) and 212 controls were studied. The mean age of the two groups was 62 years. More than half the subjects (59.8% in the case group and 51.9% in the control group) were NAT2 slow acetylators. The odds ratio for colorectal cancer was 1.38 (95% CI: 0.90-2.12) in slow acetylators. Although the number of women was small (n = 76 in the case group), the cancer risk was found to be lower in intermediate (W/Mx) acetylators [odds ratio (OR): 0.55, 95% confidence interval (95% CI): 0.29-1.02]. This difference was not observed in men (OR: 0.56, 95% CI: 0.16-2.00). Among NAT2 fast acetylators (W/W or W/Mx), meat consumption more than 3 times a week increased the risk of colorectal cancer (OR: 2.05, 95% CI: 1.01-4.16). In contrast, cigarette smoking increased the risk of CRC among slow acetylators (OR: 1.97, 95% CI: 1.02-3.79).

CONCLUSION: The risk of CRC was higher among fast acetylators who reported a higher meat intake. Slow NAT2 acetylation was associated with an increased risk of CRC.

Key words: N-acetyltransferase 2; Polymorphism; Colorectal cancer

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in the world. In Brazil, 13 310 new cases in men and

14 800 in women are estimated to occur in 2010[1].

N-acetyltransferase 2 (NAT2) is an enzyme found in a large number of organs such as the lungs, colon, breast, prostate, and liver. The expression of this enzyme suggests that it plays a key role in the protection against reactive molecules resulting from environmental insults not only in the liver but in all target tissues[2]. The *NAT2* gene is located in the chromosome 8p22 region and has no introns. The gene contains an 870-bp open reading frame and encodes a protein of 290 amino acids[3]. It is an important phase Ⅱ enzyme that catalyzes the acetylation of aromatic and heterocyclic amines and hydrazines present in carcinogenic compounds and medicines. Individuals can be divided into three different phenotypes based on the acetylation activity of NAT2: fast, intermediate, and slow. These phenotypes are determined by single nucleotide polymorphisms in NAT2[4].

Some NAT2 polymorphisms have been consistently associated with a reduction in acetylation activity (e.g. $T^{341}C$). The functional state of the phenotype is due to the impairment of protein translation or stability. No changes in mRNA levels are detected. For several polymorphisms, the classification as "fast" or "slow" is not final[5].

The probability of developing cancer depends on the natural response of each organism to different aggressive agents. Humans present different susceptibilities to carcinogens[6,7]. This difference in susceptibility to various environmental aggressors is related to genetic polymorphisms[8].

Studies have associated meat consumption with an increased risk of CRC[9]. Red meat, especially meat that is well done, is a source of chemical carcinogens such as heterocyclic aromatic amines, polycyclic aromatic hydrocarbons, and other products. The fast acetylation genotype is probably related to larger amounts of metabolic activators of heterocyclic aromatic amines when compared to the slow NAT2 acetylation genotype. Metabolic activators are transported to colorectal tissues through the bloodstream, causing DNA damage and mutations in tumor suppressor genes involved in the carcinogenesis of CRC[10].

The association between genetic polymorphisms in the *NAT2* gene and CRC has been studied extensively; however, the results are not conclusive, possibly because of ethnic differences and differences in the lifestyle and number of patients studied. The proportion of fast and slow acetylation phenotypes varies markedly depending on ethnicity and geographic origin[11]. Thus, there is an urgent need for studies investigating the distribution of NAT2 genotypes in different countries.

The aim of the present study was to investigate NAT2 polymorphisms in Brazilian patients from São Paulo.

MATERIALS AND METHODS

A case-control study involving 147 patients with CRC and 212 healthy subjects was carried out between March 2008 and December 2009. All patients were born in Brazil and were treated at the Oncology Division, Department of Gastroenterology, University Hospital, Universidade Federal de São Paulo (UNIFESP). The study was approved by the Ethics Committee of UNIFESP and all patients signed an informed consent form.

The patients answered a questionnaire regarding food habits and food frequency, whether they were current or former cigarette smokers, and their pattern of alcohol consumption.

Peripheral blood was collected for genomic DNA extraction. The *NAT2* gene polymorphisms were investigated by the polymerase chain reaction (PCR)-restriction fragment length polymorphism genotyping technique.

DNA extraction

Leukocyte DNA was extracted from peripheral venous blood collected with ethylenediaminetetraacetic acid using the Invisorb® Spin Blood Mini Kit.

Analysis of NAT2 genetic polymorphisms

The genotypes of the NAT2 polymorphism were analyzed as described previously[9]. Genomic DNA was amplified using the following primers: 5'-GGAACAAATTGGACTTGG-3' and 5'-TCTAGCATGAATCACTCTGC-3'. After amplification, the PCR product was digested with KpnI (M1 allele), BamHI (M3 allele) and MspI/AluI (M4 allele), and with TaqI (M2 allele). The digestion products were separated on agarose gels stained with ethidium bromide, and were then visualized under UV light[15]. The W/W and W/MX genotypes were classified as conferring the fast acetylation phenotype and the Mx/MX genotype as conferring the slow acetylation phenotype.

Statistical analysis

The Student *t*-test was used for the comparison of age between groups. Differences in the polymorphisms between the two groups were determined by the χ^2 test. This test was also used to compare clinical variables between NAT2 genotypes and alleles in the group of cancer patients. The association between the risk of developing cancer and these variables was assessed by calculating the odds ratio (OR) and 95% confidence interval (95% CI). A P value < 0.05 was considered to be statistically significant and a P value of 0.05 to 0.10 was considered to be marginally significant.

RESULTS

A case-control study including 147 patients with CRC and 212 healthy controls was conducted to determine whether NAT2 genetic polymorphisms are associated with the development of this disease. The characteristics of the cancer patients and controls are shown in Table 1. No difference in age or gender was observed between groups. Among the 147 patients with cancer, 90 (61.2%) had colon cancer and 57 (38.8%) had rectal cancer. According to the TNM classification, most patients were stage Ⅱ (44.2%) or stage Ⅲ (26.5%).

Four different NAT2 alleles were found, including the wild type (WT) and the M1, M2 and M3 polymorphisms. The M4 allele was not detected. Among healthy control

Table 1 Characteristics of the patients in both groups *n* (%)

Parameters	Patients	Control	*P*
Age (yr, ± DP)	61.9 (13.6)	62.0 (13.4)	0.96[a]
⩽ 50	30 (20.4)	31 (14.6)	0.196[b]
> 5	17 (79.5)	181 (85.4)	
Gender			
Male	71 (48.3)	85 (40.1)	0.15[b]
Female	76 (51.7)	127 (59.9)	
Tumor site			
Colon	90 (61.2)		
Rectum	57 (38.8)		
Stage			
I	23 (15.6)		
II	65 (44.2)		
III	39 (26.5)		
IV	20 (13.6)		

[a]*t* test; [b]χ^2 test.

subjects, the observed genotype frequency of the NAT2 polymorphisms were consistent with the expected frequency of the Hardy-Weinberg equilibrium (P = 0.56), suggesting that the distribution of NAT2 genotypes is adequate in the cancer-free population.

The slow acetylation phenotype predominated in the two groups (59.8% in the case group and 51.9% in the control group). No significant differences in the frequency of the NAT2 polymorphisms were observed between groups (Table 2).

The odds ratio for CRC was 1.38 (95% CI: 0.90-2.12) in slow acetylators. The M1 allele was the most frequent allele in the two groups, with a frequency of 45% in the control group and of 44.5% in the case group, followed by the WT allele (28% in the control group and 25.1% in the case group).

No significant association was observed between NAT2 polymorphism and acetylation phenotype or tumor site. Comparison of patients in TNM stage I or II *vs* stage III or IV showed a higher frequency of the slow acetylation phenotype in stage I and II patients (60.1%).

Analysis of red meat intake showed that half of the subjects consumed red meat more than 3 times a week. Subjects with the fast acetylation phenotype who consumed meat more than 3 times a week presented an increased risk of CRC (OR: 2.05, 95% CI: 1.01-4.16) (Table 3). With respect to cigarette smoking, the number of ex-smokers was marginally higher in the cancer group. Cigarette smoking increased the risk of CRC among slow acetylators (Mx/Mx) (OR: 1.97, 95% CI: 1.02-3.79) (Table 4).

DISCUSSION

CRC is one of the most common cancers. Almost 70% of CRC patients are diagnosed at age 65 years or older[12]. Most of the subjects studied here were women (n = 76, 51.7%), and the mean age was 61.9 years. These findings agree with data published by the Brazilian National Cancer Institute[1].

According to the Annual Report to the Nation on the Status of Cancer, prostate cancer is the most frequent cancer among men, followed by lung, colon and rectal cancer, except for Latin America where the incidence of CRC is slightly higher than that of lung cancer. Among women, the most frequent cancer is breast cancer, followed by lung cancer and CRC[13].

Variations in the frequency of NAT2 genotypes/phenotypes among different populations and ethnic groups have been reported in several studies carried out in different regions around the world. In this respect, a high frequency of the slow acetylator phenotype is observed in populations of European and African descent. Other populations are characterized by a high frequency of fast acetylation phenotypes, such as Japanese, Chinese and Amerindians[14-16].

In the present study, the slow acetylation phenotype was slightly more frequent in the two groups, although the difference was not statistically significant. However, when divided by gender, the fast acetylation phenotype tended to be more common among women. The M1 allele was the most frequent allele in the two groups (45% in the control group and 44.5% in the case group), followed by the WT allele (28% in the control group and 25.1% in the case group). The M4 allele was not detected. These data are consistent with the literature, which indicates a difference in the frequency of the WT, M1 and M4 alleles between Caucasians and Africans, whereas the frequency of the M2 and M3 alleles is similar. The M4 allele is detected at a rate of less than 1% in Caucasians, whereas its frequency is 18% in the African population[9,17,18].

No significant difference in NAT2 polymorphism or acetylation phenotype was observed between tumor sites. Analysis according to TNM stage showed that the slow acetylation phenotype was more frequent among stage I or II patients (60.1%) compared to stage III or IV. This finding might be explained by the fact that the NAT2 fast acetylation phenotype activates carcinogens and produces mutations more quickly, resulting in aggressive tumors. However, these findings should be analyzed carefully because of the small number of patients participating in the present study.

For a long time, genetic susceptibility to cancer has been attributed to xenobiotic exposure. This view was mainly due to the fact that the molecular mechanisms involved in carcinogenesis were not known. However, this view has changed over recent years with the advances in molecular biology. It is now known that exposure to xenobiotics and the development of cancer vary among individuals because of variations that occur at the molecular level which, in turn, are under genetic control[19]. In recent studies, lifestyle habits including alcohol and tobacco use and dietary habits (i.e. adequate protein and fiber intake) have been associated with gene mutations in an attempt to obtain more consistent results regarding cancer risk factors and prognosis. Although currently available data are controversial due to ethnic differences and differences in lifestyle, this has been the best approach to better understand carcinogenesis at the molecular level.

Smoking has been associated with several types of cancer other than lung cancer, including cancer of the oral cavity, pancreas, and kidney[20]. A recently published meta-

Table 2 Distribution of N-acetyltransferase 2 polymorphism and the risk of cancer

Genetic polymorphism NAT2[a]	Cancer, *n* (%)	Control, *n* (%)	*P*	OR (95% CI)
All	147	212		
Mx/Mx	88 (59.8)	110 (51.9)	0.19	1
W/Mx	44 (30.0)	83 (39.1)		0.66 (0.42-1.05)
W/W	15 (10.2)	19 (8.9)		0.99 (0.47-2.05)
Slow	88 (59.8)	110 (51.9)	0.17	1.38 (0-2.12)
Fast	59 (40.1)	102 (48.1)		
Female	76	127		
Mx/Mx	42 (55.2)	58 (45.6)	0.07	1
W/Mx	23 (30.2)	58 (45.6)		0.55 (0.29-1.02)
W/W	11 (14.4)	11 (8.6)		1.38 (0.55-3.48)
Slow	42 (55.2)	58 (45.6)	0.24	1.47 (0.83-2.60)
Fast	34 (44.7)	69 (54.3)		
Male	71	85		
Mx/Mx	46 (64.8)	52 (61.1)	0.67	1
W/Mx	21 (29.5)	25 (29.4)		0.95 (0.47-1.92)
W/W	4 (5.6)	8 (9.4)		0.57 (0.16-2.00)
Slow	46 (64.7)	52 (61.1)	0.76	0.86 (0.45-1.65)
Fast	25 (35.2)	33 (38.9)		

[a]Homozygous individuals with genotype W/W, and heterozygote W/Mx, are grouped into fast acetylation phenotype, while homozygous Mx/Mx are grouped in slow acetylators. The percentages of data are in parentheses. NAT2: N-acetyltransferase 2; OR: Odds ratio; 95% CI: Confidence interval.

Table 3 Comparison between meat intake and risk of of cancer in rapid acetylator patients *n* (%)

	Cancer	Control	*P*	OR	Lower[95% CI]/upper
All	59	102			
High meat intake[1]	44 (74.5)	60 (58.8)	0.06	2.05	1.01/4.16
Low meat intake[2]	15 (25.5)	42 (41.2)			

[1]More than 3 time per week; [2]Less than 3 times a week. OR: Odds ratio; 95% CI: Confidence interval.

Table 4 Correlation between genotypes and risk for cancer in smokers or ex-smokers

Genetic polymorphism	Cancer, *n* (%)	Control, *n* (%)	*P*	OR (95% CI)
NAT2[a]				
All	65	87		
Mx/Mx	40 (61.5)	39 (44.8)	0.09	1
W/Mx	22 (33.8)	39 (44.8)		1.82 (0.92-3.60)
W/W	3 (4.6)	9 (10.3)		3.08 (0.77-12.22)
Slow	40 (61.5)	39 (44.8)	0.06	1.97 (1.02-3.79)
Fast	25 (38.5)	48 (61.0)		

[a]Homozygous individuals with genotype W/W, and heterozygote W/Mx, are grouped into fast acetylation phenotype, while homozygous Mx/Mx are grouped in slow acetylators. NAT2: N-acetyltransferase 2; OR: Odds ratio; 95% CI: Confidence interval.

analysis reported a strong association between smoking and the development of CRC[12]. However, smoking is currently not recognized as a risk factor for CRC by the International Agency for Research on Cancer (IARC) or the US Surgeon General[12]. Sørensen *et al*[21] studied the association between NAT1 and NAT2 polymorphisms, smoking, meat consumption and CRC risk in 379 cancer patients and 769 healthy subjects. In that study, only the NAT1 polymorphism affected cancer risk. However, the NAT1 and NAT2 fast acetylation phenotype increased the risk of CRC among patients who smoked more cigarettes, suggesting that N-acetylation status affects the relationship between smoking and CRC risk.

In the present study, most subjects in the two groups had never smoked, but the rate of ex-smokers was higher in the case group than in the control group. Once diagnosed with cancer, individuals tend to break old habits that may affect the prognosis and treatment of the disease even if it is not possible to reverse the previous damage. The slow acetylation phenotype was more frequent among smokers of the case group, suggesting an increased risk of cancer (OR: 1.97, 95% CI: 1.02-3.79) in subjects with this phenotype. A higher frequency of the slow acetylation phenotype among patients with lung and bladder cancer has been demonstrated in other studies. Carcinogens present in tobacco are metabolized by NAT enzymes and activation of these enzymes is reduced in slow acetylators, thus increasing the risk of cancer[22].

An association between red meat consumption and a higher risk of CRC has been reported in case-control studies, prospective epidemiological studies and in a recent meta-analysis[23]. The last study suggested that this increased risk is due to the production of polycyclic aromatic hydrocarbons and heterocyclic amines when meat is cooked at high temperatures[24].

Tamer *et al*[25], studying 125 patients with CRC and 82 healthy subjects, observed an association between NAT2 polymorphisms and cancer development. In that study, high protein intake was found to be correlated with an increased risk of colon cancer (OR: 1.73, 95% CI: 1.10-3.07). Patients with the NAT2 * 14A (M4 allele) fast acetylation phenotype and high meat intake presented an increased risk of CRC (OR: 3.03, 95% CI: 1.56-5.86). In the present study, the risk of cancer was higher among patients consuming meat more than 3 times per week (OR: 1.65, 95%

CI: 1.05-2.61). The risk of CRC was increased among patients presenting the fast acetylator genotypes (W/W or W/Mx) and a high frequency of meat intake (OR: 2.05, 95% CI: 1.01-4.16).

Heterocyclic amines are formed when meat is cooked by the condensation of creatinine with amino acids. The NAT2 fast acetylation phenotypes are more readily able to convert N-hydroxy heterocyclic amines into carcinogens, a fact predisposing to cancer. Thus, heterocyclic amines require metabolic activation to induce DNA mutations and to initiate carcinogenesis. After N-oxidation, N-hydroxy aromatic and heterocyclic amines are activated (*via* O-acetylation) by NAT to acetoxy intermediates, which react spontaneously with DNA to form adducts[26,27]. The increased cancer risk observed in patients with the NAT2 fast acetylation phenotype and high meat consumption suggests that heterocyclic amines mediated by metabolic activation of NAT2 fast acetylation might be important carcinogens and increase the risk of cancer.

In conclusion, the proportion of subjects with the slow acetylation phenotype was high in this study. No association was observed between the risk of CRC and NAT2 polymorphisms. However, the slow acetylation phenotype increased the risk of CRC in smokers and the fast acetylation phenotype increased this risk among subjects with high red meat intake.

COMMENTS

Background

Colorectal cancer (CRC) is considered the fourth leading cause of cancer worldwide and is one of the most common malignancies in the West. The probability of developing cancer depends on the natural response of each organism to different exposures from various aggressors. Humans have different susceptibilities to different carcinogens and lifestyle may be a risk factor for cancer. The interaction between diet, alcoholism, cigarette smoking, obesity and physical inactivity can lead to its development.

Research frontiers

The HAAs (present in red meat, tobacco, *etc.*) are bioactivated through N-oxidation by the enzymes CYP1A2 in the liver or by CYP1A1/CYP1B1 in extra hepatic tissues. The products of this oxidation are the N-hydroxy-N, which in turn suffer the bioactivation in the liver by O-acetylation of N-acetyltransferase (NAT) enzymes (mainly NAT2) and sulfotransferase. NAT2 fast acetylation individuals may have larger amounts of metabolic activators of HAA than slow acetylation NAT2 subjects that can be transmitted to the colorectal tissues through the bloodstream, causing DNA damage and mutations.

Innovations and breakthroughs

Previous studies linking meat consumption and colorectal cancer have concluded that diets rich in meat consumption increase the risk of this disease. In the case of red meat, the degree of cooking can be a source of exposure to chemical carcinogens such as heterocyclic amines, nitrosamines and other products. This study aimed to identify the distribution of NAT2 gene polymorphism in a Brazilian population from São Paulo correlating these polymorphisms and the phenotypes of NAT2 acetylation with the consumption of red meat, alcohol and cigarette smoking and the colorectal cancer risk.

Applications

The proportion of people with the phenotype of fast and slow acetylation varies considerably depending on the ethnicity and geographic origin. This variability lead to the investigation into the frequency of NAT2 genotypes and the association of this polymorphism with colorectal cancer in a Brazilian population of São Paulo. The relationship between the mechanism of acetylation by NAT2 and susceptibility to colorectal cancer may possibly screen patients who have a higher risk of developing this disease.

Terminology

The NAT2 is an important phase Ⅱ enzyme that catalyzes the acetylation of heterocyclic aromatic amines and hydrazines, which include carcinogenic compounds and drugs. Based on the activity of NAT2 acetylation, subjects are divided into three different phenotypes: fast, intermediate and slow. Single nucleotide polymorphisms in NAT2 determine this phenotype.

Peer review

The authors examined the phenotypes and polymorphisms of NAT2 acetylation on a case-control study, involving the eating habits and lifestyle as risk modifiers of colorectal cancer development. The results indicated that cigarette smokers with slow acetylation phenotype had an increased risk of developing colorectal cancer and individuals with fast acetylation phenotype intake of red meat increased the risk of developing the disease. These results are interesting because the study of NAT2 acetylation may identify individuals with a higher risk of developing CRC in relation to environmental factors and diet.

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BRIEF ARTICLE

Identification of patients at-risk for Lynch syndrome in a hospital-based colorectal surgery clinic

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Abstract

AIM: To determine the prevalence of a family history suggestive of Lynch syndrome (LS) among patients with colorectal cancer (CRC) followed in a coloproctology outpatient clinic in Southern Brazil.

METHODS: A consecutive sample of patients with CRC were interviewed regarding personal and family histories of cancer. Clinical data and pathology features of the tumor were obtained from chart review.

RESULTS: Of the 212 CRC patients recruited, 61 (29%) reported a family history of CRC, 45 (21.2%) were diagnosed under age 50 years and 11 (5.2%) had more than one primary CRC. Family histories consistent with Amsterdam and revised Bethesda criteria for LS were identified in 22 (10.4%) and 100 (47.2%) patients, respectively. Twenty percent of the colorectal tumors had features of the high microsatellite instability phenotype, which was associated with younger age at CRC diagnosis and with Bethesda criteria ($P < 0.001$). Only

5.3% of the patients above age 50 years had been previously submitted for CRC screening and only 4% of patients with suspected LS were referred for genetic risk assessment.

CONCLUSION: A significant proportion of patients with CRC were at high risk for LS. Education and training of health care professionals are essential to ensure proper management.

Key words: Colorectal cancer; Family history; Hereditary cancer; Lynch syndrome; Microsatellite instability phenotype

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INTRODUCTION

Family history of colorectal cancer (CRC) is a clinically significant risk factor and may be reported by up to 15% of all patients with the disease. Lynch syndrome (LS, OMIM: # 120435), also called hereditary non-polyposis colorectal cancer syndrome (HNPCC), is the most common inherited colon cancer predisposition syndrome. It is an autosomal dominant syndrome caused by germline mutations in the mismatch repair (MMR) genes *hMLH1*, *hMSH2*, *hMSH6* and *PMS2*. The syndrome accounts for 2%-3% of all CRC diagnoses and for 5%-9% of the diagnoses of endometrial cancer in patients under age 50 years[1-4]. Other extra-colonic tumors including ovarian, upper urologic tract, gastric, small bowel, biliary/pancreatic and brain cancers have been described at an increased frequency in families with LS[5]. The cumulative lifetime risk of cancer varies depending on geographic/environmental factors and the age-related incidence of each tumor type[6-8]. Furthermore, the cancer spectrum in families affected with the syndrome varies significantly based upon the DNA MMR gene mutated and the specific mutation[9,10].

Determining the prevalence of LS among patients with CRC is an important public health issue. Affected patients have an increased risk for second primary cancers and their identification can lead to specific screening and intervention recommendations for patients and their at-risk relatives[11-13].

Cancer family history is an important tool to identify at-risk patients and families. The Amsterdam criteria, initially including only CRC and later all tumors of the LS cancer spectrum, define clinical diagnosis of LS and are in themselves an indication for MMR mutation testing. However, even the revised Amsterdam Ⅱ criteria have a relatively low sensitivity (< 80%) which has turned out to be a major limitation for LS diagnosis. More recently, the Bethesda guidelines were developed to identify a larger proportion of MMR mutation carriers (Table 1)[14].

Multiple other strategies for identifying individuals with LS have been proposed, including predictive mathematical models to define prior probabilities of carrying a germline MMR mutation, family history instruments and routine testing of CRCs from patients with specific risk factors (e.g. age < 50 years), but the effectiveness of these approaches continues to be debated and has limited applicability in clinical practice[9,10].

In this study, we aimed to determine the prevalence of a family history suggestive of LS among patients with CRC followed in a coloproctology outpatient clinic of a University Hospital in Southern Brazil, and to identify all potential LS patients who should be referred for genetic counseling. Also, we investigated the frequency of tumors with histopathologic features suggestive of microsatellite instability (MSI) and whether screening recommendations were correctly modified according to the risk identified.

MATERIALS AND METHODS

Ethics

This study was approved at the Institutional Ethics Committee (GPPG-HCPA) under the number 05-257.

Patients

All consecutive patients with a diagnosis of CRC who had an appointment in the outpatient Coloproctology clinic of Hospital de Clínicas de Porto Alegre (HCPA), in Porto Alegre, Southern Brazil, from December 2005 to December 2006, were considered for participation in this study. From a total of 250 patients seen in this period, 212 unrelated patients with adenocarcinoma of the colon and rectum and without a previous diagnosis of inflammatory bowel disease were invited and agreed to participate in the study. After signature of informed consent forms, data regarding personal and family cancer history, pathology reports and additional relevant clinical and/or surgical information were collected. Information collected included types of cancer and age at diagnosis, presence of multiple (synchronous or metachronous) tumors, type and periodicity of colorectal screening, tumor histology, clinical and histological stage of tumors (Dukes and TNM Staging System), family history of cancer (first, second and third degree). Tumor diagnoses in family members were confirmed by medical records and/or death certificates whenever possible.

"Early-onset" colorectal or endometrial cancer was defined as cancer diagnosed before the age of 50 years. All other extra-colonic tumors described in the LS (ovarian, upper urologic tract, gastric, small bowel, biliary/pancre-

Table 1 Clinical criteria for Lynch syndrome

Name	Criteria	Sensitivity[1]	Specificity[1]
Amsterdam	Amsterdam criteria Ⅰ	61.0%	67.0%
	Three or more relatives with colorectal cancer, one of whom is a first-degree relative of the other two, FAP should be excluded		
	Colorectal cancer involving at least two generations		
	One or more colorectal cancer cases diagnosed before the age of 50		
	Amsterdam criteria Ⅱ	78.0%	61.0%
	Three or more relatives with histologically verified LS-associated cancer (colorectal cancer, cancer of the endometrium, small bowel, ureter, or renal pelvis), 1 of whom is a first-degree relative of the other 2; FAP should be excluded		
	Colorectal cancer involving at least two generations		
	One or more cancer cases diagnosed before the age of 50		
Revised bethesda	At least one of the following features	90.9%	77.1%
	Bethesda 1: Colorectal cancer diagnosed in a patient under the age of 50		
	Bethesda 2: Presence of synchronous or metachronous colorectal cancer, or other LS-associated tumors[2], regardless of age		
	Bethesda 3: Colorectal cancer with the MSI-H histology[3] under the age of 60		
	Bethesda 4: Colorectal cancer in one or more first-degree relatives with an LS-related tumor, with one of the cancers under the age of 50		
	Bethesda 5: Colorectal cancer in two or more first- or second-degree relatives with LS-related tumors, regardless of age		

[1]Data on sensitivity and specificity of Amsterdam criteria from Syngal *et al*[14] and Revised Bethesda from Piñol *et al*[46]; [2]Lynch syndrome (LS)-associated tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, brain tumors, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome, and carcinoma of the small bowel; [3]Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern. FAP: Familial adenomatous polyposis; MSI-H: Microsatellite instability-high.

atic and brain) were considered in the pedigree analyses[5]. Colorectal surveillance was considered appropriate when colonoscopy was performed by or after age 50 years in patients with no history of CRC in first- or second-degree relatives, and in those with a positive family history, when performed 10 years before the earliest diagnosis of CRC in the family, and every 1-2 years thereafter.

The criteria used to identify patients with LS or at-risk for the syndrome included the Amsterdam Ⅰ and/or Ⅱ criteria for clinical diagnosis[5,15] and the Bethesda Revised Criteria[16] for a potential diagnosis.

Statistical analysis

SPSS version 16.0 was used for data handling and statistical analyses. For descriptive analysis, categorical variables were described by their absolute and/or relative frequencies and quantitative variables were expressed as mean ± SD. For analytical statistics, the existence of an association between categorical variables was examined using χ^2. The Student's *t* test was used to determine the significance between different ages at diagnoses among two independent groups. A difference with a *P* value of less than 0.05 was considered significant.

RESULTS

Clinical information on the 212 patients studied is summarized in Table 2. Mean age of the patients at recruitment was 62.33 years (range: 24-99 years, SD = 12.8 years), and 113 (53.3%) were female. Of the 212 patients, 45 (21.2%) were diagnosed with CRC under the age of 50 years and the mean age at first CRC diagnosis was 59.8 years (range: 20-99 years, SD = 13.1 years). Approximately

Table 2 Sample description by criteria for Lynch syndrome (*n* = 212)

Lynch syndrome criteria	*n* (%)
Amsterdam	22 (10.4)
Amsterdam Ⅰ	16 (7.6)
Amsterdam Ⅱ	6 (2.8)
Bethesda (at least 1 of the 5 criteria)	100 (47.2)
Bethesda (2 or more of the criteria)	41 (19.3)
Bethesda by criteria	
Bethesda 1	45 (21.2)
Bethesda 2	17 (8.0)
Bethesda 3	27 (12.7)
Bethesda 4	23 (10.8)
Bethesda 5	36 (17.0)

5.2% of the patients had a synchronous or metachronous colorectal tumor (*n* = 11) and the mean age at diagnosis in this group was 51.6 years (range: 36-80 years, SD = 13.1 years), lower than the mean age in patients without metachronous colorectal tumor (59.3 years, range: 20-99 years, SD = 12.8 years), as expected (*P* = 0.051). Two patients (0.9% of the sample) were diagnosed with familial adenomatous polyposis.

The age at diagnosis of the first cancer varied from 20 to 86 years (mean = 58.9 years; median = 59 years; SD = 12.9 years). A second primary cancer was present in 33 patients: CRC in 11 (33.3%), endometrial in 2 (6.1%), breast in 2 (6.1%), prostate in 6 (18.2%). The age at diagnosis of the second primary varied from 39 to 99 years (mean = 66.5 years, SD = 13.2 years). One patient was diagnosed with four different primary tumors: two colon cancers at ages 36 and 55 years, endometrial can-

Table 3 Features of the 223 colorectal tumors in the 212 probands[1]

Feature	*n* (%)
Tumor site	
Ascending colon	22 (9.9)
Transverse colon	12 (5.4)
Descending colon	11 (5.0)
Rectosigmoid	158 (71.1)
Other (cecum, unspecified site)	19 (8.6)
Total	222 (100.0)
Missing data	1
Differentiation	
Well differentiated	20 (10.0)
Moderately differentiated	157 (78.1)
Poorly differentiated	24 (11.9)
Total	201 (100.0)
Missing data	22
Mucinous feature	15 (6.7)
Total	223 (100.0)
Missing data	0
MSI-high phenotype[2]	42 (21.3)
Total	197 (100.0)
Missing data	26
Dukes stage (*n* = 195)	
A	6 (3.1)
B	76 (39.0)
C	87 (44.6)
D	26 (13.3)
Total	195 (100.0)
Missing data	28

[1]Cases with missing data were excluded from the analysis; [2]Cases included all patients with microsatellite instability-high phenotype, independent of age.

cer at 45 years, bladder cancer at 61 years and renal carcinoma at 62 years; all of them confirmed with pathology records. This patient was later found to carry a germline mutation in *hMLH1* (data not shown).

Among the 212 unrelated probands, family history of cancer up to second-degree relatives was observed in 60.4% of patients with early-onset and 52.4% of those with late-onset CRC diagnoses. Twenty-nine percent of the patients reported a family history of CRC and almost 50% fulfilled criteria for LS: 22 (10.4%) fulfilled Amsterdam I and/or II criteria and 100 (47.2%), Revised Bethesda Criteria. In the families of probands with LS criteria, 180 relatives had cancer and the most frequent primary sites were: colon and rectum (43.4%), lung (8.9%), breast (7.8%), stomach (6.7%), endometrium (6.7%), ovaries (3.9%) and prostate (3.4%). As expected, this distribution was different in patients without LS criteria: colon and rectum (30.8%), lung (12.3%), breast (12.3%), stomach (4.6%), uterus (1.5%), ovarian (0%) and prostate (12.3%). About 2.5% of the patients had relatives with multiple primary tumors: one case was diagnosed with uterine and ovarian cancer, and another with colorectal, esophageal and gastric cancer.

Family history of breast cancer was present in 6.4% of the sample with available information (11/171). Among the patients with at least one of the Bethesda criteria, 12% had a family history of breast cancer, compared to 7.1% among patients with none of the criteria ($P = 0.248$).

Table 4 Comparison of different features among patients with and without the microsatellite instability-high phenotype (*n* = 197[1]) *n* (%)

Features	MSI-high phenotype (*n* = 42)	Non MSI-high phenotype (*n* = 155)	*P*
Age at diagnosis < 50 yr	18 (42.9)	29 (18.7)	0.001
Family history of colorectal cancer	15 (35.7)	45 (29.0)	0.404
Presence of Revised Bethesda criteria[16]	42 (100)	56 (36.1)	< 0.001
Presence of Amsterdam Ⅱ criteria	6 (14.3)	15 (9.7)	0.391
Second primary tumors	3 (7.1)	8 (5.2)	0.620
Early stage at diagnosis	11 (26.2)	64 (41.3)	0.079

[1]15 patients were excluded due to missing data on tumor histology. MSI: Microsatellite instability.

In the overall sample, 22 (9.9%) patients had a tumor in the ascending colon and 21.3% had colorectal tumors with histology suggestive of MSI-high (MSI-H) phenotype (Table 3). The clinical features of patients with and without this phenotype are shown in Table 4. As expected, MSI-H histological features were more commonly seen in patients with CRC diagnosed at a younger age (18 patients from a total of 42, $P = 0.001$) and in patients who fulfilled the Bethesda criteria (in all 42 patients, $P < 0.001$). Opposite to what was expected, in a significant number of tumors from individuals fulfilling Amsterdam criteria, histological features suggestive of MSI-H phenotype were not encountered.

Of the 212 charts reviewed, 17% had the family history previously documented. Furthermore, when the previously documented family histories were compared to the pedigrees obtained during patient interview for this study, there was concordance of data in only 56.6% of cases. On the other hand, of the 100 patients with a family history of cancer and fulfilling Bethesda criteria for LS, 57.0% had their family history previously collected and/or reported in the chart by clinicians or surgeons, but a clinical suspicion of LS was not documented in the chart in any of these cases. Only 4% of the patients with clinical criteria for LS were referred for genetic cancer risk evaluation.

Finally, 5.3% of patients with indications for population-based screening by colonoscopy starting at age 50 years had been submitted at least once for colonoscopy before the diagnosis of CRC. Among the 100 patients at risk for LS, only 4.1% had been offered surveillance colonoscopy previously.

DISCUSSION

As in most familial cancer syndromes, early age of onset and multiplicity of cancers have been considered hallmarks of LS. In registry-based series, the mean age at first CRC is about 45 years, compared to 65 years for sporadic CRC, and some LS patients present with CRC in their twenties. Similarly, the mean age of endometrial cancer is about 50 years, which is about 10 years younger than the average age of sporadic endometrial cancer.

As our knowledge of the influences of genetics on cancer risk has increased, so has the need to improve physicians' awareness of the importance of familial cancer history and its proper recording in the medical chart[17]. Although there is no consensus about the correct method for obtaining information on cancer family history, and using it as a screening tool[18], general practitioners usually collect the family history data at the time of registration[19] and different groups have reported screening the adult population for increased genetic risk of cancer using postal questionnaires[20,21]. Unfortunately, however, health professionals in the first line of patient contact are usually unaware of how and when to contact genetic services. Many specialists, especially coloproctologists and gastroenterologists, have a key role in identifying high-risk patients; the ability to suspect a patient to be at risk for a cancer predisposition syndrome is crucial for a rapid diagnosis and to ensure appropriate care.

Nearly 30% of patients in our study reported a positive family history of CRC, which is much higher than observed positive CRC family history in the general population: approximately 9.0%[22]. Our findings also indicate that the hereditary CRC phenotype can be easily identified in outpatient coloproctology units, using a systematic approach after proper training of the staff, as reported previously[20,21].

Surprisingly, a high number of patients fulfilling Amsterdam criteria were found in our sample (22/212, 10.4%), significantly higher than previously reported in the Brazilian population. Viana *et al*[23], reviewing 311 medical records of CRC patients from São Paulo, Brazil, found a frequency of 1.3% in families with Amsterdam criteria. The reason for such a high incidence remains to be explained. One potential limitation of this study is the fact that the outpatient coloproctology clinic from which the patients derive is located in a tertiary care university hospital, and reference center for many diseases in the region. Thus, a higher percentage of high-risk patients, including those at risk for hereditary cancer may exist in this setting than in other general hospitals.

An additional point to consider is the fact that this institution has, since 2001, one of the few cancer risk evaluation clinics in Southern Brazil. This fact, however, would ideally be associated with high indices of correct identification and referrals of the hereditary cases, which was not observed in most cases. Most patients evaluated in our study did not have an accurate family history assessment in their charts, (description of family history was present in the charts of only 57.0% of potential LS patients). Moreover, only 4% of patients with LS criteria were referred for genetic counseling, suggesting that even when detailed family cancer history is obtained, it may not be granted the necessary importance. Previous investigations have already reported significant gaps in the documentation of family cancer history in medical charts. Analyzing data from the Direct Observation of Primary Care study, Medalie *et al*[24] found that only 40% of 2333 audited charts documented the presence or absence of a family history of breast or colon cancer. Even when family cancer history is obtained, its interpretation seems to be problematic as referrals often focus on rarer, hereditary cancer syndromes, neglecting cases of average and moderate risk individuals. Tyler and Snyder found a similar deficiency in the referral process among family physicians: from 10 patients considered at moderate or high risk, only 3 had been identified and none had been referred for cancer genetic consultation[25].

Also, although previous studies have shown a clear benefit for surveillance colonoscopy in patients with suspected or proven LS[26,27], in our series it was not common practice. Several factors could be contributing to this observation: patient's refusal, physician omission of adequate familial risk assessment and or referral and/or limited access to screening examinations. Our results are in agreement with those previous studies, considering that only 5% of the patients evaluated were undergoing proper screening. The identification of high-risk precursor lesions is considered critically important and colonoscopy screening for individuals with LS is recommended to begin at age 25 years, or 10 years younger than the earliest diagnosis of CRC in the family, whichever comes first, every 1-2 years[28].

Multiplicity of cancers is a hallmark of LS and according to the literature about 10% of identified Lynch patients have more than one cancer by the time of diagnosis. Although CRC is the most common, other frequent findings include cancers of endometrium, ovary, stomach, small bowel, pancreas, hepatobiliary system, renal pelvis, ureter and glioblastoma. Approximately 20%-40% of patients have been reported to develop metachronous CRC after initial resection if a subtotal colectomy is not performed[29]. Similarly, clustering of more than one Lynch-associated cancer (colorectal and uterine) in an individual patient should raise suspicion of LS. In our population, among all CRC cases, approximately 15% of patients had a second primary cancer. The most common second primary was CRC (metachronous or synchronous), endometrium, breast and prostate.

The most common extra-colonic tumor in LS is endometrial carcinoma, which develops in up to 70% of women who are mutation gene carriers[30]. Thus, the presence of endometrial cancer in the family history of an individual with CRC, a feature encountered in 12 (6.7%) of the patients studied here, should always raise a suspicion of LS. When the endometrial cancer is diagnosed under the age of 50 years, the probability of LS is particularly high. This has been confirmed by a high rate of MMR deficiency (up to 40%) in women with early-onset endometrial cancer[31,32]. Consistent with this, the National Comprehensive Cancer Network (NCCN) has recently revised its guidelines and currently recommends annual surveillance of the endometrium for LS families, as well as MMR deficiency investigation for all women diagnosed with endometrial cancer under age 50 years[28].

There is some debate with regard to whether prostate or breast cancers might be part of the LS, since some studies have found a high frequency of such tumors among HNPCC families[33,34]. Oliveira Ferreira *et al*[22] found a 26.5% frequency of breast cancer in families fulfilling Amsterdam criteria from São Paulo, Southeastern

Brazil, a higher rate than found in general population-based studies, which could suggest existence of a subset of mutations in this region that are also associated with a higher breast cancer risk. In our study, we found breast cancer at a frequency of approximately 9% in families fulfilling Amsterdam criteria. Although it is common to see LS pedigrees with breast cancer, most genetic and immunohistochemical studies on familial breast cancers have not found any strong relationship with the MMR system deficiency[35,36]. Recently, a case report of a Lebanese family with LS found a *MSH2* gene defect in a breast cancer tumor of early-onset, suggesting that it could be involved in accelerated breast carcinogenesis[37]. However, more studies are needed to define such association.

Different studies have shown that CRC in LS differs from typical sporadic CRCs in location, histology, and natural history. Also, it usually displays findings that are suggestive of MSI, such as intense lymphocytic infiltrates, extensive areas with poorly differentiated tissue and mucinous histology[38]. Overall, 20.3% of the tumors evaluated in this series demonstrated one or more histological features suggestive of MSI-H, similar to previous studies[39]. In the last few years, it has been suggested that histology could be useful in selecting CRC patients for molecular testing for LS[40-43], since limiting testing to patients with MSI-H histology could reduce the burden of molecular testing by 60% compared with testing all patients who meet the Revised Bethesda Criteria. More importantly, it could help identify LS among patients with late onset and no cancer family history[39].

Interestingly, among patients who fulfilled the Amsterdam criteria, only 28.6% presented tumors with features of MSI-H phenotype. This is somewhat surprising and could indicate that in this group of patients, the strong family history of CRC may not be related to abnormalities in the MMR system, and that there could be an increased prevalence of what has been called in the literature the Familial Colorectal Cancer Type X[44]. Recently, Abdel-Rahman *et al*[45] analyzed the molecular features of the tumors in CRC patients with MMR germline mutations and in sporadic CRC. They concluded that tumors from the MMR gene-negative group exhibited a novel molecular pattern characterized by a paucity of changes in the common pathways to CRC, which could be associated with non MSI-H histology. Molecular studies with this population are now being carried out in order to test such hypothesis.

Large population-based studies have shown that a family history of CRC in first-degree relatives is associated with an increased risk of CRC. In this study we have shown that a significant proportion of patients diagnosed with CRC and followed in an outpatient clinic of a university hospital in Southern Brazil have either a significant family history of cancer or pathology features suggestive of LS. Our findings underscore the importance of adequate familial risk assessment and, also, of considering MSI-H pathology features in the identification of at-risk patients. Education and training of physicians is essential to ensure that hereditary cancer patients and families are identified and properly referred for genetic counseling and long-term cancer screening programs. The reason for the high prevalence of patients at risk for LS in our population requires further investigation.

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COMMENTS

Background

The incidence of colorectal cancer (CRC) is currently rising in Southern Brazil. Patients at-risk for the hereditary forms of the disease are diagnosed at younger ages, and have an increased risk for second colorectal tumors and extra-colonic malignancies. Identification of these patients can lead to specific screening and intervention recommendations.

Research frontiers

In this study, the authors describe high prevalence of patients at-risk for Lynch syndrome (LS) in a coloproctology clinic and results support the principle that education and training of health care professionals are essential to ensure proper management of these individuals.

Innovations and breakthroughs

The study draws attention to the high frequency of potential LS patients in a coloproctology clinic in Southern Brazil. It reinforces the importance of correct identification of these cases and suggests further investigations of the origins for these observations.

Applications

Description of the high frequency of hereditary CRC cases in this setting is the first step to demonstrate that adequate familial risk assessment is fundamental to identify at-risk patients.

Peer review

Study was undertaken very well. It's a well written paper. It highlights the importance of identifying high risk groups in order to carry on with surveillance in CRC.

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BRIEF ARTICLE

Single center experience of capsule endoscopy in patients with obscure gastrointestinal bleeding

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Abstract

AIM: To identify optimum timing to maximize diagnostic yield by capsule endoscopy (CE) in patients with obscure gastrointestinal bleeding (OGIB).

METHODS: We identified patients who underwent CE at our institution from August 2003 to December 2009. Patient medical records were reviewed to determine type of OGIB (occult, overt), CE results and complications, and timing of CE with respect to onset of bleeding.

RESULTS: Out of 385 patients investigated for OGIB, 284 (74%) had some lesion detected by CE. In 222 patients (58%), definite lesions were detected that could unequivocally explain OGIB. Small bowel ulcer/erosions secondary to Crohn's disease, tuberculosis or non-steroidal anti-inflammatory agent use were the commonest lesions detected. Patients with overt GI bleeding for < 48 h before CE had the highest diagnostic yield (87%). This was significantly greater (P < 0.05) compared to that in patients with overt bleeding prior to 48 h (68%), as well as those with occult OGIB (59%).

CONCLUSION: We established the importance of early CE in management of OGIB. CE within 48 h of overt bleeding has the greatest potential for lesion detection.

Key words: Capsule endoscopy; Gastrointestinal bleeding

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INTRODUCTION

Obscure gastrointestinal bleeding (OGIB) is responsible for about 5% of all gastrointestinal (GI) bleeding[1]. Although it represents a small proportion of patients with GI bleeding, OGIB continues to be a challenge because of delay in diagnosis and consequent morbidity and mortality. In recent times, capsule endoscopy (CE) and device-assisted enteroscopy have established their position in the management algorithm for OGIB, and have had a significant impact on the outcome. CE is superior to push enteroscopy[2,3], small bowel follow-through[4] and computed tomography (CT)[5] for detection of the bleeding source in the small bowel. There is however concern about sensitivity of CE in the setting of ongoing GI bleeding, due to possible visualization of blood limiting the interpretation.

Most published reports on CE in OGIB are limited to small groups of patients[6-8]. Although most differentiate between occult and overt GI bleeding when analyzing diagnostic yield, they do not identify the optimum time for performing CE in the overt GI bleeding group. We evaluated the diagnostic yield of CE in identifying the source of bleeding in OGIB. We further analyzed our patients to answer the question regarding proper timing of performing CE in overt OGIB, to maximize diagnostic yield. To the best of our knowledge, the present study of 385 patients with OGIB is the largest single-center experience of CE in OGIB.

MATERIALS AND METHODS

Patient selection

Patients who presented with evidence of GI bleeding at the clinic or emergency department were enrolled in the present study after negative upper GI endoscopy and full-length colonoscopy. Between August 2003 and December 2009, 505 patients underwent CE at our center. 345 patients underwent the procedure as inpatients, whereas 160 were outpatients. Of these, 385 (76.2%) had CE for OGIB (Figure 1). Patients with OGIB were further classified into three categories: (1) persistent overt bleeding, i.e. bleeding documented within 48 h at the time of first evaluation; (2) recent overt bleeding, i.e. last episode of bleeding > 48 h prior to the first evaluation; and (3) obscure occult bleeding, i.e. anemia associated with positive fecal occult blood without overt bleeding.

CE procedure

The GIVEN Video Capsule system (Given Imaging, Yoqneam, Israel) was used with M2A/SB capsules. The reader system was updated during the study period from Rapid 3 to Rapid 5. The real time viewer (Given Imaging) was used during the final 6 mo of the study. Patients were allowed a light diet on the previous evening and were prepared by using an oral purge at night (2 L polyethyleneglycol-based solution or 90 mL sodium phosphate mixed with 350 mL lime-based drink followed by 1 L water). Patients swallowed the capsule between 09:00 and 11:00 h, and were maintained on nil by mouth for the next 4 h. Six patients had swallowing difficulty and had their capsule delivered into the stomach using an endoscope with the help of an AdvanCE device. Patients with known diabetes mellitus and history of vomiting that was suggestive of gastroparesis were given two doses of intravenous metoclopramide (10 mg) during the study. Intravenous metoclopramide was also given to three of 40 patients who were found to have their capsule in the stomach on real time study, even at 2.5 h after capsule ingestion. The recorder of CE was disconnected only after the battery stopped blinking at 8-11 h after capsule ingestion. Only one procedure had technical difficulty with the capsule not becoming active after removal from the container, and had to be replaced with another capsule. All other patients had a smooth examination.

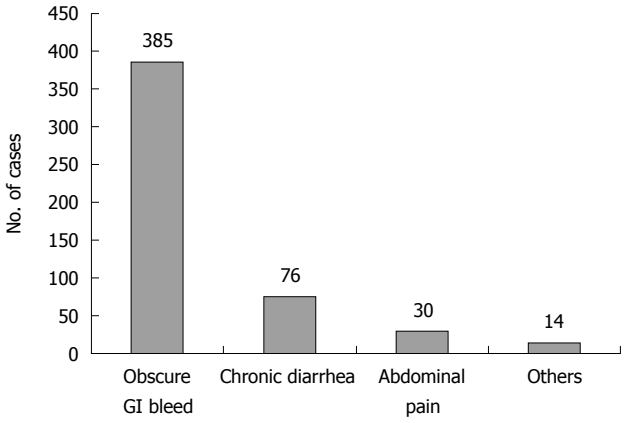

Figure 1 Indication for capsule endoscopy (n = 505).

Image interpretation

The interpretation of images was done by a single gastroenterologist (MKG) after initial detailed evaluation by a trained technician who had been involved in > 50 000 GI endoscopic procedures. Findings were categorized as definite, suspicious or negative as follows: (1) definite: lesions with definite bleeding potential that clearly explained the clinical situation; (2) suspicious: mucosal lesions identified, but bleeding could not be conclusively attributed to them, or blood was seen in the small intestine without any definite lesion being identified; and (3) negative: no lesion or bleeding identified, or incomplete study.

Follow-up

Patients were asked to note evacuation of the capsule, and those who were uncertain or concerned, as well as those who were suspected to have retained the capsule, as suggested by capsule image interpretation, were followed by serial X-ray/fluoroscopic screening at weekly intervals. Patients were also followed up with medical therapy (such as treatment of Crohn's disease, institution of antitubercular therapy, or antihelminthic therapy), surgical therapy (for tumors or bleeding ulcers) or enteroscopic evaluation (ulcers, polyps, or bleeding angiodysplasia), depending on the CE results. Those with negative CE were followed up with expectant treatment or surgery with preoperative enteroscopy. The study was approved by our institutional review board.

Statistical analysis

Statistical methods included χ^2 analysis for comparison of the positive diagnostic yield of CE between the three different categories of OGIB. $P < 0.05$ was considered to be statistically significant.

RESULTS

OGIB was the commonest (76.2%) indication for CE during the study period (Figure 1). Out of the 385 patients with OGIB, 275 (71%) were male with age ranging from 12 to 80 years. One hundred and one patients (26.2%) had a negative examination, either because no obvious lesion was found until the small intestine (n = 93) or because

Table 1 Positive/suspicious lesions detected by capsule endoscopy in patients with obscure gastrointestinal bleeding (*n* = 284)

	Definite (*n* = 222)	Suspicious (*n* = 62)	Total (*n* = 284)
Ulcers/erosions	156	32	188
Tumor	48	0	48
AVM	18	7	25
Worms	0	8	8
Only blood	0	15	15

101 patients had negative capsule endoscopy. AVM: Arteriovenous malformation.

the progress was slow (*n* = 8). Of the eight patients with slow progress of the capsule, six had diabetes mellitus. Of the 101 patients with negative CE, nine underwent laparotomy because of recurrent/persistent bleeding, and in eight, some lesion was found at preoperative enteroscopy (Meckel's diverticulum, 1; small-intestinal ulcers, 3; and angiodysplasia, 4). One patient had negative laparotomy. Of the remaining 92 patients in this group, only 52 were available for a follow-up of 1 year and none had any significant bleeding.

Two hundred and eighty-four patients (73.8%) had some lesion detected at CE. Although 272 of these lesions were located in the small intestine, 12 (ulcers/erosions, 8; angiodysplasia, 3; and gastric fundal tumor, 1) had findings in the stomach/duodenum that were missed at pre-CE gastroscopy. Two hundred and twenty-two patients (57.7%) were considered to have definite lesions that could explain OGIB, whereas another 62 (16.1%) had lesions that were suspicious but bleeding could not be completely attributed to these findings. The latter included 32 patients with small ulcers and erosions, seven with doubtful angiodysplasia, eight with worms (3 roundworm, 3 whipworm, and 2 hookworm) and 15 patients with blood in the jejunum or ileum, without any underlying lesion being identified. Four of these patients with evidence of bleeding but no underlying lesions underwent mesenteric angiography that also detected bleeding, but no obvious pathology was found and bleeding stopped with supportive treatment alone.

As demonstrated in Table 1, the 222 patients with definite lesions at CE included ulcers/erosions in 156, tumors in 48, and angiodysplasia in 18. Among those with tumors, two had multiple polyps that were suggestive of Peutz-Jeghers syndrome. It was difficult to characterize ulcers/erosions, but at least 12 were considered to be tubercular (based on abdominal CT scan ± fine needle aspiration cytology and follow-up), 42 patients were considered to have Crohn's disease (based on fissuring serpiginous ulcers with cobble-stone appearance, or histology from tissue obtained at enteroscopy or surgery), and 12 were nonsteroidal anti-inflammatory drug (NSAID)-induced. Of the 18 patients with arteriovenous malformation (AVM), four underwent double-balloon enteroscopy (DBE), three had successful treatment with argon plasma coagulation, and the others were put on hormonal therapy/tranexamic acid.

Figure 2 shows the distribution of patients according

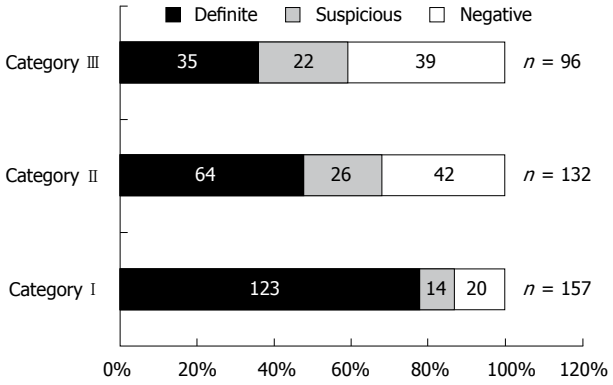

Figure 2 Diagnostic yield of capsule endoscopy according to category of bleeding (*n* = 385)

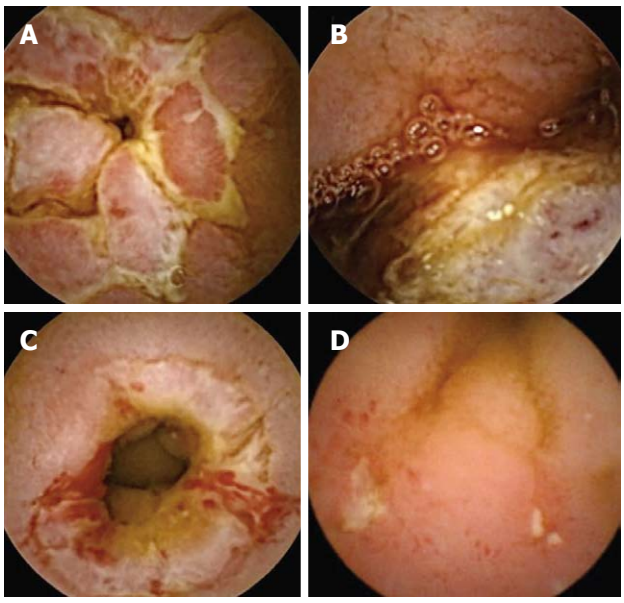

Figure 3 Capsule endoscopy images. A: Cobble-stone appearance characteristic of Crohn's disease; B: An ileal tumor; C: Circumferential ulcer with narrowing and minor ongoing bleeding in a patient with Crohn's disease; D: Small intestinal ulcers in a patient with tuberculosis.

to category of bleeding. In patients with ongoing bleeding (Category Ⅰ), positive findings were seen in 87.2% (definite in 78.3%), whereas in patients with previous overt bleeding (Category Ⅱ), it was 68.2% (definite in 48.5%), and in the occult OGIB group (Category Ⅲ), only 59.3% (definite in 36.4%). The ability of CE to identify a definite bleeding source was significantly higher for Category Ⅰ than Category Ⅱ and Ⅲ patients ($P < 0.05$), but there was no significant difference in the diagnostic yield when comparing Category Ⅱ and Ⅲ patients (Figure 3).

Capsule retention was noted in six of 385 patients (1.6%). All these patients had strictures in the small bowel either due to tuberculosis or Crohn's disease, which were not suspected or identified prior to CE. Three of these patients underwent surgery, two were lost to follow-up, and one refused surgery and continues to have capsule retention, but has been asymptomatic during follow-up of 9 mo.

DISCUSSION

CE has gained widespread clinical acceptance in the diagnostic algorithm of OGIB[9,10]. As in our study, OGIB is now the leading indication for CE in most centers around the world. Prior to the introduction of CE, barium examination, push enteroscopy and angiography were the principle diagnostic tools for OGIB. The diagnostic yield of these tests has been shown to be unequivocally inferior to CE in several studies. Recently, DBE has been used in several centers for diagnosis of OGIB. However, diagnostic yield of CE has been found to be significantly higher compared to a single DBE examination done via the oral or anal route (137/219 *vs* 110/219, OR: 1.67, 95% CI: 1.14-2.44, $P < 0.01$)[11].

The reported yield of CE in OGIB varies widely. Previous studies have shown that detection rates for the source of bleeding varies from 38% to 93%, and is in the higher range for those with overt OGIB[9,10]. This is further influenced by subjective interpretation of positive findings. To address this issue in our study, we divided positive findings into definite and suspicious groups. Although the overall diagnostic yield in our study cohort was 73.8%, a definite lesion that could explain OGIB was obtained in only 57.7%. A recently published study by Hindryckx *et al*[8] which considered CE to be positive only when lesions with sufficient bleeding potential were detected, reported a similar diagnostic yield of 59.8%.

Recent studies have indicated that the optimum timing of CE in OGIB is within the first few days, with acceptable maximum duration of 2 wk[13-17]. In a recently reported series of 260 patients with OGIB, the yield was 87% in patients with ongoing overt OGIB and 46% in those with occult OGIB[10]. In our patients, a definite lesion could be detected in 64.7% of patients with overt OGIB compared to 36.4% in patients with occult OGIB ($P < 0.01$). Moreover, the diagnostic yield of CE was significantly higher in patients who had evidence of bleeding within 48 h of CE (Category Ⅰ) compared to those who had remote overt bleeding (Category Ⅱ) [123/157 (78.3%) *vs*. 64/132 (48.5%) OR: 3.84, 95% CI: 2.31-6.41, $P < 0.01$, respectively]. The diagnostic yield of CE was not significantly different when comparing patients with remote overt OGIB (> 48 h before CE) (Category Ⅱ) and those with occult OGIB (Category Ⅲ). This highlights the importance of using CE early in the diagnosis of OGIB. Pennazio *et al* also have found the highest yield in patients with ongoing GI bleeding, and therefore have recommended ordering CE earlier in the setting of overt OGIB. There have been concerns in the past regarding the possibility of blood obscuring proper visualization of the mucosa in patients who are actively bleeding. A recent study that has compared massively bleeding patients with chronic overt OGIB has found a similar positive yield in both groups [59.18% (29/49) and 52.69% (137/260), respectively][18]. These results demonstrate that, for optimum diagnostic efficacy, CE should be done within 48 h of bleeding in patients with OGIB.

The definition of a positive finding on CE continues to be ambiguous. For the purpose of this study, nonspecific mucosal changes such as red spots, focal erythema and fold thickening, were not considered to be clinically significant. Ulcers and erosions were included as positive findings in this series if they could completely or partially account for the GI bleeding. Moreover, active bleeding without definite lesions was described as a suspicious finding in this study. The commonest lesion detected in our patients was small-bowel ulcers and erosions, followed by tumors and AVM. A previous study from India also has documented small-bowel ulcers to be the commonest lesion detected by CE in OGIB[19]. A definite underlying etiology could be established in 66 (42.3%) out of the 156 patients with ulcers/erosions, and nearly two-thirds (42/66) of them were considered to be due to Crohn's disease.

The current study has several limitations. In the first place it is a retrospective single-center study. However data was obtained from forms filled at the time of CE, thereby minimizing data collection bias. Secondly this study does not offer long-term follow-up of the patients and hence makes it impossible to draw a strong conclusion as to the fate of CE-negative OGIB. Moreover a large proportion of ulcers/erosions could not be characterized due to inherent difficulty of obtaining small bowel mucosal biopsies. However, this study enabled us to analyze positivity rates, nature of lesions and optimum timing of CE in a relatively large cohort of subjects comprising of a heterogeneous population of patients with OGIB. Although the data demonstrates the diagnostic utility of early CE in OGIB it does not reflect whether an early diagnostic intervention and unequivocal identification of a bleeding source affects clinical outcome in this group of patients.

In summary, high diagnostic yield, relative safety and tolerability have established CE as an important diagnostic tool for OGIB. In this large cohort of OGIB patients, we demonstrate that small bowel ulcer/erosions secondary to Crohn's disease, tuberculosis or NSAID-use are the commonest lesions responsible for OGIB in this part of the world. Moreover, the diagnostic yield is significantly affected by the timing of CE and studies done within 48 h of an episode of overt bleed have the greatest potential for detecting a definite lesion.

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COMMENTS

Background

The advent of video capsule endoscopy (CE) has resulted in a paradigm shift in the approach to the diagnosis and management of patients with obscure gastrointestinal bleed (OGIB). With increasing global availability of this diagnostic tool, it has now become an integral part of the diagnostic algorithm for OGIB in most parts of the world. However there is scant data on optimum timing of CE for maximizing diagnostic yield. OGIB continues to be a challenge because of delay in diagnosis and consequent morbidity and mortality.

Research frontiers

Previous studies have shown that capsule endoscopy detection rates for the source of bleeding varies from 38% to 93%, being in the higher range for those with overt OGIB. Results in most studies are further influenced by subjective interpretation of "positive findings". The authors classified our patients depending on time since last episode of bleed and looked at diagnostic yield in the different groups with the aim to identify a time-frame to guide clinical decision-making on when to do a capsule endoscopy in this cohort of patients.

Innovations and breakthroughs

Diagnostic yield is significantly affected by the timing of CE and studies done within 48 h of an episode of overt bleed have the greatest potential for detecting a definite lesion. The diagnostic yield of CE was not significantly different when comparing patients with overt OGIB prior to 48 h of CE and those with occult OGIB. This highlights the importance of obtaining a CE early in the diagnosis of OGIB.

Applications

This article suggests a potential benefit of doing a capsule endoscopy within 48 h of an episode of bleed in patients with OGIB in terms of increasing chances of detecting a bleeding source. However, further studies are needed to determine if early detection of lesion translates into better patient outcome.

Peer review

The paper provides well-collected information about early detection of small intestinal lesions by CE in obscure GI bleed. Research should be aimed at finding if early detection results in improved patient outcome.

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BRIEF ARTICLE

Serum magnesium concentration in children with functional constipation treated with magnesium oxide

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Abstract

AIM: To determine whether hypermagnesemia recently reported in adult patients possibly develops in children with functional constipation taking daily magnesium oxide.

METHODS: We enrolled 120 patients (57 male and 63 female) aged 1-14 years old (median: 4.7 years) with functional constipation from 13 hospitals and two private clinics. All patients fulfilled the Rome Ⅲ criteria for functional constipation and were treated with daily oral magnesium oxide for at least 1 mo. The median treatment dose was 600 (500-800) mg/d. Patients were assessed by an interview and laboratory examination to determine possible hypermagnesemia. Serum magnesium concentration was also measured in sex- and age-matched control subjects (n = 38).

RESULTS: In the constipation group, serum magnesium concentration [2.4 (2.3-2.5) mg/dL, median and interquartile range] was significantly greater than that of the control group [2.2 (2.0-2.2) mg/dL] (P < 0.001). The highest value was 3.2 mg/dL. Renal magnesium clearance was significantly increased in the constipation group. Serum magnesium concentration in the constipation group decreased significantly with age (P < 0.01). There was no significant correlation between the serum level of magnesium and the duration of treatment with magnesium oxide or the daily dose. None of the patients had side effects associated with hypermagnesemia.

CONCLUSION: Serum magnesium concentration increased significantly, but not critically, after daily treatment with magnesium oxide in constipated children with normal renal function.

Key words: Children; Constipation; Hypermagnesemia; Magnesium oxide; Renal dysfunction

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INTRODUCTION

Magnesium-containing cathartics are used worldwide to treat chronic constipation[1-3]. Approximately 45 million Japanese patients are estimated to undergo treatment with magnesium oxide as an antacid or cathartic annually[4]. Many children with functional constipation are taking these drugs for long periods of time; sometimes over several years.

Hypermagnesemia is a rare clinical condition[5,6]. Most cases are iatrogenic and due to increased intake of magnesium, which occurs after intravenous administration of magnesium[7,8] or oral ingestion of high doses of magnesium-containing antacids or cathartics[9-12]. Magnesium homeostasis is dependent mainly on gastrointestinal absorption and renal excretion. The kidney is the principal organ involved in magnesium regulation. Renal magnesium excretion is very efficient, because the thick ascending limb of Henle has the capacity to reject completely magnesium reabsorption under conditions of hypermagnesemia[5,6], and therefore, hypermagnesemia commonly arises in patients with renal dysfunction.

In 2008, the Ministry of Health, Labour and Welfare (MHLW) of Japan reported 15 adult patients with hypermagnesemia, including two cases of death due to oral ingestion of magnesium oxide from April 2005 to August 2008[4]. Although most of these elderly patients with constipation had dementia, schizophrenia, or renal dysfunction, the MHLW has recommended measuring the serum level of magnesium in patients who regularly use magnesium oxide.

It is now important for pediatricians to know whether hypermagnesemia can develop in children without abnormal renal function after administration of a common or high dose of magnesium oxide. The purpose of this study was to determine serum magnesium concentration in children with functional constipation treated with daily magnesium oxide.

MATERIALS AND METHODS

Patients

We enrolled 120 patients (57 male and 63 female) aged 1-14 years with functional constipation from 13 hospitals and two private clinics in Japan. At entry, all patients fulfilled the Rome Ⅲ criteria for functional constipation, which meant that they had at least two of the following characteristics: fewer than three bowel movements weekly; more than one episode of fecal incontinence weekly; large stools in the rectum shown by digital rectal examination or palpable on abdominal examination; occasional passage of large stools; retentive posturing and withholding behavior; and painful defecation. All patients had been treated for at least 1 mo with daily magnesium oxide as an oral laxative. The medication was given once daily or in split doses. The dose was dependent on the patient's condition.

Children with known organic causes of constipation, including Hirschsprung disease, spinal and anal congenital abnormalities, previous colon surgery, inflammatory bowel disease, allergy, metabolic or endocrine diseases, renal dysfunction, and severe neurological disability were excluded from the study. Patients with poor drug compliance were also excluded.

In each patient, we recorded the date of initiation of constipation, daily dose of magnesium oxide, and duration of treatment. We also determined whether the patient had symptoms that could be side effects of hypermagnesemia, such as vomiting, nausea, thirst, blushing, feeling of exhaustion, or somnolence. The laboratory examinations carried out were as follows: serum level of magnesium, calcium, phosphorus, blood urea nitrogen (BUN), and creatinine concentration. Urinary concentrations of magnesium and creatinine were also measured. Magnesium clearance and fractional excretion of magnesium (FEMg) were calculated as follows: Magnesium clearance = urine magnesium (mg/dL)/urine creatinine (mg/dL); FEMg = urine magnesium (mg/dL)/serum magnesium (mg/dL) × serum creatinine (mg/dL)/urine creatinine (mg/dL).

Table 1 Subject characteristics and serum magnesium concentrations

	Control group (n = 38)	Constipation group (n = 120)	P value
Age (yr)	5.5 (2.0-10.8)	4.7 (3.0-6.8)	NS[a]
Gender, male (%)	63.2	47.5	NS[b]
Serum magnesium concentration (mg/dL)	2.2 (2.0-2.3)	2.4 (2.3-2.5)	< 0.001[a]

[a]Mann-Whitney U test; [b]χ^2 test. NS: Not significant.

Control group

Serum magnesium concentrations were also measured in the control group which comprised 38 children (24 male and 14 female) aged 1-15 years who visited the Department of Pediatrics at Gunma University Hospital, and were without any history of hematological disease, tumor, heart failure, metabolic or endocrine diseases, renal dysfunction, or severe neurological disability. None of these children were treated with magnesium oxide.

Statistical analysis

Laboratory values, duration of treatment, and daily dose of magnesium oxide are shown as the median and interquartile ranges. Statistical significance of differences was tested by χ^2 test or Mann-Whitney U test, as appropriate. Spearman's rank correlation coefficients were calculated for the correlation between the serum level of magnesium and age, duration of treatment, and drug dose. $P < 0.05$ was regarded as significant. All analyses were carried out using SPSS for Windows (SPSS statistics 17.0).

RESULTS

Subject characteristics and serum magnesium concentrations are shown in Table 1. In the constipation group, the median treatment duration with magnesium oxide was 1.3 (0.4-2.6) years and median daily dose was 600 (500-800) mg/d; 33 (25-45) mg/kg per day. After administration of magnesium oxide, the outcome of constipation was investigated in 83 patients. Bowel habits in all patients were improved, and 75% of patients were stable. However, 11% of children had fewer than three bowel movements weekly, 28% of them had withholding behavior, and 34% had painful defecation during the follow-up period. None of the patients still had overflow-incontinence.

The median serum magnesium concentration in the

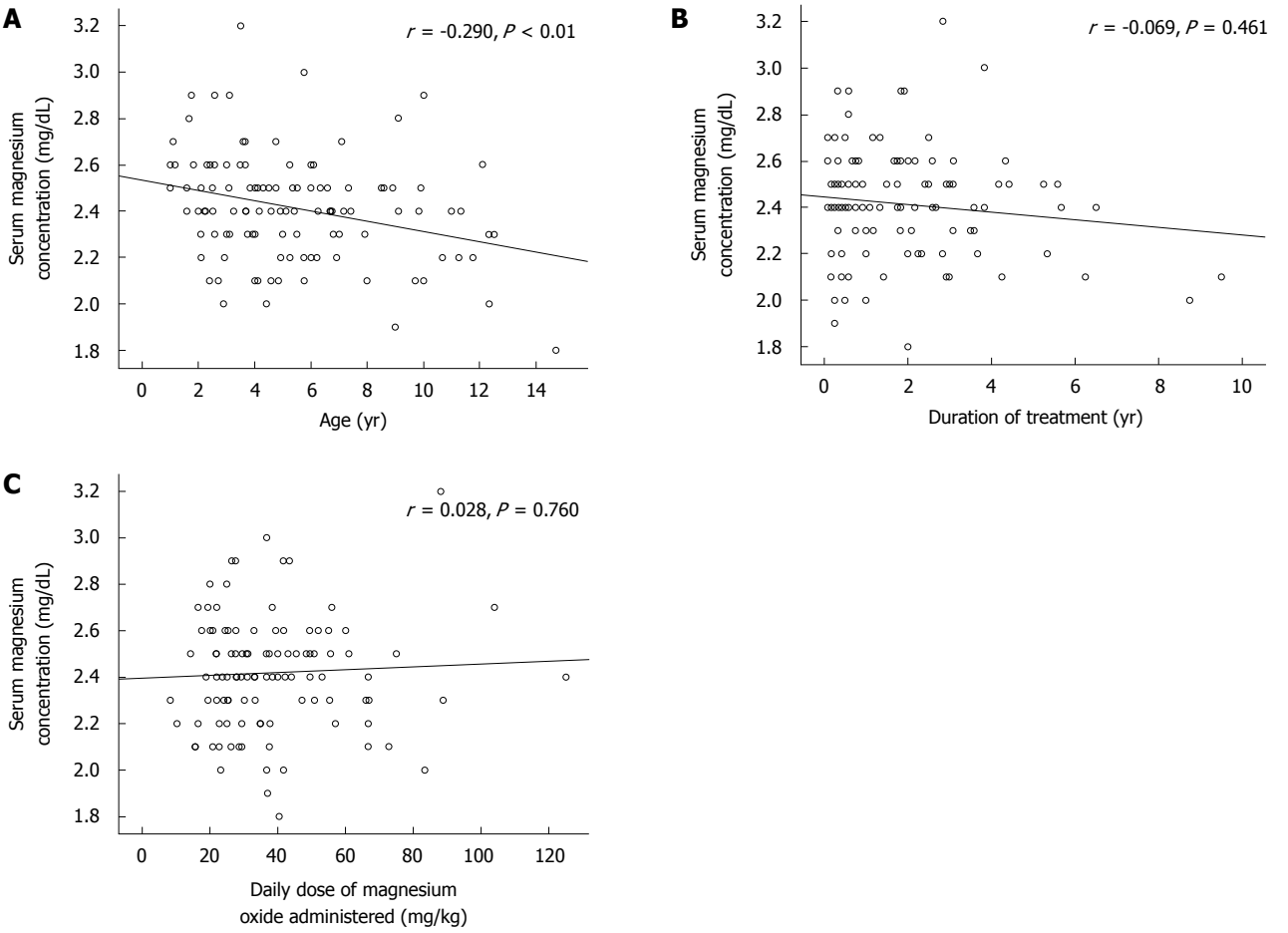

Figure 1 Correlation between serum level of magnesium and age (A), and duration of treatment with magnesium oxide (B), and dose of magnesium oxide (C).

constipation group was significantly greater than that in the control group (Table 1). The highest magnesium concentration was 3.2 mg/dL in a 3.5-year-old patient treated with 1320 mg/d; 88 mg/kg per day magnesium oxide for 2.8 years.

The median urinary magnesium to creatinine ratio in the constipation group was significantly elevated compared with that reported previously [0.23 (0.15-0.37) (n = 76) *vs* 0.15 (0.12-0.20) (n = 16), $P < 0.05$)[13]. The median FEMg in the constipation group was 0.03 (0.02-0.05).

Serum magnesium concentration in the constipation group decreased significantly with age ($P < 0.01$) (Figure 1A). There was no significant correlation between the serum level of magnesium and duration of treatment (Figure 1B). The treatment dose had no effect on serum magnesium level (Figure 1C).

Serum level of calcium [9.9 (9.5-10.2) mg/dL], phosphorus [5.2 (4.8-5.6) mg/dL], creatinine [0.3 (0.3-0.4) mg/dL], and BUN [13.0 (10.8-15.5) mg/dL] were not abnormal in any of the patients. None of the patients had side effects associated with hypermagnesemia.

DISCUSSION

In 2008, the MHLW of Japan reported that 15 patients aged 32-98 years (median, 71 years) who had been treated with magnesium oxide developed severe side effects of magnesium toxicity, such as hypotension, bradycardia, electrocardiographic changes (atrial fibrillation), loss of consciousness, coma, respiratory depression, and cardiac arrest. The serum magnesium concentration in two fatal cases was 20.0 mg/dL and 17.0 mg/dL. As a result of these reported cases, the MHLW has recommended that the serum concentration of magnesium in subjects on continuous magnesium therapy should be determined[4].

Most cases of hypermagnesemia in adults result from large intravenous doses of magnesium[7,8] or from excessive enteral intake of magnesium-containing cathartics[9-12]. Symptomatic hypermagnesemia is likely to occur in patients with renal dysfunction. In fact, 10 of the 15 Japanese patients reported as cases of hypermagnesemia by the MHLW had renal dysfunction.

Several cases of hypermagnesemia from enteral magnesium intake in patients with normal renal function have been reported previously[14-17]. It is known that non-renal risk factors for hypermagnesemia are age, gastrointestinal tract disease, and administration of concomitant medications, particularly those with anticholinergic and narcotic effects[18]. Five elderly Japanese patients without renal dysfunction had intestinal necrosis, severe constipation, and abnormal abdominal distention with intestinal expansion, and these abdominal risk factors could have increased the serum concentration of magnesium.

Hypermagnesemia has also been reported in pediatric

practice[14,19]. These case reports include a 14-year-old girl without renal dysfunction who was taking magnesium hydroxide because of severe constipation[14], and a 2-year-old boy with neurological impairments who was taking 2400 mg/d of magnesium oxide administered as part of a regimen of megavitamin and megamineral therapy[19]. These cases developed increased serum magnesium levels as high as 14.9 mg/dL and 20.3 mg/dL, respectively.

Magnesium oxide is commonly used in patients with chronic constipation; however, not only has the optimum dose for children not been established, but there has been no study to evaluate the concentration of serum magnesium after oral administration of magnesium oxide. The aim of the present study was to determine the serum magnesium concentration in pediatric cases receiving magnesium cathartics for chronic constipation.

In our study, the median serum magnesium concentration was 2.4 mg/dL in the constipation group, which was significantly greater than that in the control group (2.2 mg/dL). Thirty patients (25%) in the constipation group and none in the control group had a serum magnesium concentration greater than the maximum value of the normal range in healthy Japanese children (2.6 mg/dL). The high critical limit of serum magnesium concentration has been reported as 4.9 ± 2.0 mg/dL in adults and 4.3 ± 1.1 mg/dL in children[20]. The highest value in our study was 3.2 mg/dL, and none of our patients reached the critical limit of serum magnesium concentration or developed symptoms due to hypermagnesemia. The median urinary magnesium to creatinine ratio in the constipation group was significantly elevated compared with that in healthy subjects, which suggests that serum magnesium level is regulated by an increase in renal excretion in those children with normal renal function, and is maintained within its appropriate range.

In our study, serum magnesium level in constipated children treated with magnesium oxide, but not in the control children, decreased significantly with age. No correlation was found between duration of treatment or daily dose of magnesium oxide and serum magnesium concentration. These data are consistent with those reported by Woodard *et al*[21] They reported that the increase in serum magnesium concentration in 102 adults who received multiple doses of magnesium citrate did not correlate with the quantity of magnesium administered[21]. Elderly patients are at risk of magnesium toxicity as kidney function declines with age, but it is not clear whether young children have a higher risk of hypermagnesemia. Alison *et al*[22] reported on a 6-week-old infant who had increased serum magnesium level (14.2 mg/dL) and life-threatening apnea due to 733 mg/d magnesium hydroxide that was used to treat constipation. Brand *et al*[23] and Humphrey *et al*[24] reported on premature infants with hypermagnesemia following antacid administration in order to decrease the risk of gastrointestinal hemorrhage. One infant had an increased serum magnesium level of 13.3 mg/dL and developed intestinal perforation. These reports indicate that infancy, prematurity or young age might be a possible risk factor for hypermagnesemia.

According to our results, we conclude that serum magnesium concentrations increase significantly after daily magnesium oxide intake, but the magnitude of the increase appears modest. Younger age, but not prolonged use of daily magnesium oxide might be a relative risk factor, and it should be determined by further studies whether serum magnesium concentration should be assessed in these subjects.

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COMMENTS

Background

Magnesium-containing cathartics are commonly used to treat chronic constipation. Although hypermagnesemia is a rare clinical condition, it can occur as a side effect of increased intake of magnesium salts.

Research frontiers

The Japanese government has recently reported fatal cases of hypermagnesemia in adults treated with magnesium oxide. In our study, serum magnesium concentrations increased significantly after daily magnesium oxide intake, but the magnitude of the increase appeared modest. Serum magnesium levels in constipated children treated with magnesium oxide, but not in the control children, decreased significantly with age. No correlation was found between duration of treatment or daily dose of magnesium oxide and serum magnesium concentration.

Innovations and breakthroughs

Recent reports have highlighted that serum magnesium concentration increases significantly, but not critically, after daily treatment with magnesium oxide in children with normal renal function.

Applications

The present study indicated the safety of daily magnesium oxide treatment for children with chronic constipation.

Peer review

The authors are to be congratulated for providing evidence of the apparent safety of a commonly used and effective therapy to treat an important health issue seen commonly in children.

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BRIEF ARTICLE

Higher parity associated with higher risk of death from gastric cancer

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Abstract

AIM: To examine the association between parity and gastric cancer (the cases are almost all premenopausal women) risk in a cohort of young parous women.

METHODS: The study cohort consisted of all women with a record of a first and singleton childbirth in the Birth Register between 1978 and 1987. We tracked each woman from the time of her first childbirth to December 31, 2008. Their vital status was ascertained by linking records to the computerized mortality database. Cox proportional hazard regression models were used to estimate hazard ratios of death from gastric cancer associated with parity.

RESULTS: There were 1090 gastric cancer deaths (85.87% of them were premenopausal) during 33686828 person-years of follow-up. The mortality rate of gastric cancer was 3.24 cases per 100000 person-years. A trend of increasing risk of gastric cancer was seen with increasing parity. The adjusted hazard ratio was 1.24 [confidence interval (95% CI): 1.02-1.50] for women who had borne two to three children, and 1.32 (95% CI: 1.01-1.72) for women with four or more births, when compared with women who had given birth to only one child.

CONCLUSION: These results suggest that higher parity may increase the risk of death from gastric cancer among premenopausal women.

Key words: Gastric cancer; Parity; Mortality; Cohort study

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INTRODUCTION

In Taiwan, gastric cancer (GC) is the fifth leading cause of cancer mortality for males and females[1]. The age-adjusted mortality rate for gastric cancer was 14.1 per 100000 among males and 7.4 among females in 2007. There is

substantial geographic variation in gastric cancer mortality within the country. In most areas, however, its mortality rate is about two-fold higher among men than women[1]. Known risk factors, such as *Helicobacter pylori* (*H. pylori*) infection, tobacco smoking, and low fruit and vegetable intake cannot entirely explain the gender difference[2].

The difference between male-to-female incidence rates is greatest during the reproductive ages, and the rates becomes more similar after menopause; it has been hypothesized that sex hormones play a role in the development or progression of gastric cancer[3]. The influence of sex hormones on gastric cancer risk is supported by the presence of steroid-hormones receptors in the gastric mucosa and gastric cancer tissues[4,5]. In rat experimental studies, there is also a greater preponderance of GC in males compared with females[6,7].

Few epidemiological studies have investigated the association between parity and gastric cancer, and results have been inconsistent. Parity was associated with increased risk of gastric cancer in four studies[8-11]. Three studies found a suggestive inverse relationship, but no significant dose-risk trend[12-14], whereas five others reported no association[15-19].

A previous study on gastric cancer in young individuals indicated that gastric cancer diagnosed in women within two years after delivery was more progressive, and proposed that pregnancy or delivery might accelerate the growth of gastric cancer[20]. Maeta *et al*[21] found that the pathological features of gastric cancer that occurred more frequently in young women were more common among pregnancy-related cases. These results suggested the need for separate analysis of pre- and postmenopausal women when examining the relationship between parity and gastric cancer risk.

Four of the above-mentioned studies, which studied the relationship between parity and gastric cancer risk, were restricted to postmenopausal women[11,12,17,18]. Only one recent Swedish study has examined the relationship between parity and gastric cancer separately for pre- and postmenopausal women[14]. Other studies did not categorize the gastric cancer cases into pre- or postmenopausal because of the lack of sufficient premenopausal gastric cancer cases to support a complete analysis.

The objective of this study was to examine the effect of parity on the risk of gastric cancer in a cohort of 1 292 462 young parous women in Taiwan, followed over a period of 31 years.

MATERIALS AND METHODS

Data source

Registration of births is required by law in Taiwan. It is the responsibility of the parents or the family to register infant births at a local household registration office within 15 d. The Birth Registration System, which is managed by the Department of the Interior, released computerized data on live births since 1978. The registration form, which requests information on maternal age, education, parity, gestational age, date of delivery, infant gender, and birth weight, is completed by the physician attending the delivery. Most deliveries in Taiwan take place in either a hospital or a clinic[22], the birth certificates are completed by physicians attending the delivery, and it is mandatory to register all live births at local household registration offices; therefore the birth registration data are considered complete, reliable, and accurate[22].

Study population

The study cohort consisted of 1 292 462 women with a record of a first and singleton childbirth in the Birth Register between January 1, 1978 and December 31, 1987. Information on any subsequent births was also retrieved from the Birth Register.

Follow-up

Each woman has her own unique personal identification number, which was used to track the women from the time of their first childbirth to December 31, 2008. Their vital status was ascertained by linking records with the computerized mortality database, identifying the date of any deaths.

Statistical analysis

We categorized parity (the number of children recorded in the last childbirth record of each woman registered during follow-up) into three categories: one, two to three, and four or more. We compared selected baseline characteristics of the cohort by parity using χ^2 tests or analysis of variance, as appropriate. Death rates were calculated by dividing the number of deaths from gastric cancer (ICD-9 code 151) by the number of person-years of follow-up. Cox proportional hazard regression models were used to estimate the hazard ratio of death from gastric cancer associated with parity. The 95% confidence intervals (CIs) for the hazard ratios were also calculated. We used two Cox proportional hazard models: an age-adjusted model and a multivariate-adjusted model, which was additionally adjusted for marital status (married, unmarried), years of schooling ($\leqslant 9$, > 9 years), and birthplace (hospital/clinic, home/other). The proportion hazards assumption was assessed for all above-mentioned variables and no violations were observed. Analyses were performed using the SAS statistical package (version 8.02, SAS Institute Inc). All statistical tests were two-sided. Values of $P < 0.05$ were considered statistically significant.

RESULTS

The study cohort was comprised of 1 292 462 primiparous women with complete information. A total of 33 686 828 person-years were observed in this study. The mean follow-up period was 26.09 (standard deviation = 3.28) years. During the follow-up period, 1090 gastric cancer deaths were recorded, yielding a mortality rate of 3.24 cases per 100 000 person-years.

Table 1 presents the baseline characteristics of the study population by parity. Compared with women who had given birth to only one child, women with four or

Table 1 Demographic characteristics of the study cohort (mean ± SD) *n* (%)

	Parity			*P*-value
	1 (*n* = 157207)	2-3 (*n* = 1000977)	4+ (*n* = 134278)	
Age at recruitment (1st birth)	26.38 ± 4.43	24.26 ± 3.22	22.44 ± 2.95	< 0.001
Marital status				
Married	146022 (92.89)	984049 (98.31)	130544 (97.22)	< 0.001
Not married	11185 (7.11)	16928 (1.69)	3734 (2.78)	
Years of schooling				
≤ 9	72090 (45.86)	544098 (54.36)	106330 (79.19)	< 0.001
> 9	85117 (54.14)	456879 (45.64)	27948 (20.81)	
Birth place				
Hospital/clinic	153167 (97.43)	970422 (96.95)	122336 (91.11)	< 0.001
Home/other	4040 (2.57)	30555 (3.05)	11942 (8.89)	

Table 2 Association between parity and hazard ratio of death from gastric cancer over a 31-year follow-up period

Parity	No. of subjects	Follow-up person-years	No. of gastric cancer (per 100000)	Age-adjusted HR (95% CI)	Multivariate-adjusted HR[1] (95% CI)
1	157207	4020271.75	128 (3.18)	1.00	1.00
2-3	1000977	26036992.42	848 (3.25)	1.23 (1.01-1.49)	1.24 (1.02-1.50)
4+	134278	3629563.83	114 (3.14)	1.33 (1.02-1.73)	1.32 (1.01-1.72)
				P = 0.030 for linear trend	*P* = 0.035 for linear trend

[1]Adjusted for age, marital status, years of schooling, and birth place. HR: Hazard ratio; CI: Confidence interval.

more children were more likely to have lower educational level, younger age at first birth, and a lower chance of being born in a hospital or clinic.

Table 2 presents the hazard ratios of gastric cancer mortality by parity. After adjustment for age at first birth, the hazard ratio for gastric cancer death was 1.23 (95% CI: 1.01-1.49) for women who had two to three children, and 1.33 (95% CI: 1.02-1.73) for women with four or more births, when compared with women who had given birth to only one child. In the multivariate-adjusted model, the hazard ratios were only slightly altered. The adjusted hazard ratio was 1.24 (95% CI: 1.02-1.50) for women who had borne two to three children, and 1.32 (95% CI: 1.01-1.72) for women with four or more births, when compared with women who had given birth to only one child. There was a significant increasing trend in the adjusted hazard ratios of gastric cancer with increasing parity (*P* for trend = 0.035).

DISCUSSION

To our knowledge, this is the largest cohort (*n* = 1292462 women) published to date to examine the relationship between parity and gastric cancer risk. In this prospective cohort study, we found a positive association between parity and gastric cancer risk. Our finding of an increased risk of gastric cancer associated with higher parity agrees with some previous studies[8-11], but not with other studies that reported the reverse effect[12-14] or no association[15-19] with parity. Pregnancy elevates serum estrogen levels by about 100 fold[23]. Increasing parity is associated with an overall increase in lifetime exposure to sex hormones. There is experimental evidence that gastric cancer carcinogenesis might be inhibited by estrogens[6,7]. Thus, if estrogens are associated with a reduced risk of gastric cancer, we would expect pregnancy to offer some protection from gastric cancer. Our data did not provide support for this hypothesis.

On the other hand, it has been reported that estrogen stimulates the growth of gastric cancer cell lines[24], and there is evidence that pregnancy or delivery might accelerate the growth of gastric cancer[20]. The mean age at death for gastric cancer was 42.90 ± 7.08 years in this study. The majority of gastric cancer deaths (85.87%) were premenopausal (using age 50 as the cut-off value[14]). Women included in this study tended to be younger (with the large majority of the gastric cancer deaths occurring before menopausal age) than in previous studies. The mean time of gastric cancer was about 14 years after last delivery (age at the birth of the last child = 28.97 ± 4.01; age at the death for gastric cancer = 42.90 ± 7.08). It is possible that these premenopausal gastric cancers were influenced by hormonal conditions caused by the actual events of pregnancy or delivery[17]. Our finding of an increased risk of gastric cancer associated with higher parity may therefore plausibly be related to a short-term increase in risk after a delivery[16]. The incidence of gastric cancer in premenopausal women is low; therefore, such effects are easily lost in overall analyses[17]. Some studies have been restricted to postmenopausal women[11,12,17,18]. To our knowledge, this is the first cohort study to indicate that a positive association between parity and gastric cancer may be only restricted to premenopausal women. However, because there is no consistent evidence to date for an association between parity and risk of death from gastric cancer, the possibility that this is a chance finding must also be considered. Clearly, more work will be needed before the influence of parity on the risk of gastric cancer is understood.

In the event of a death in Taiwan, the decedent's family is required to obtain a death certificate from the hospital or local community clinic, which then must be submitted to the household registration office to cancel the decedent's household registration. The death certificate is required to have the decedent's body buried or cremated. Death certificates must be completed by physicians in Taiwan. It is also mandatory to register all deaths at local household registration offices; thus, the death registration is reliable and complete[22]. The complete population coverage and follow-up made possible by the national identification number has left the study without selection bias. Information bias is also unlikely to be important for parity.

Taiwan is a small island with a convenient communication network. It is believed that all gastric cancer cases had access to medical care. Mortality data rather than data on inpatient cases was used to assess the association between parity and gastric cancer in this study. The mortality of a disease is a function of its incidence and fatality. Gastric cancer has been reported to have the fifth poorest five-year relative survival rate among all cancer sites[25]. Deaths from gastric cancer may therefore be regarded as a reasonable indicator of the incidence of gastric cancer.

Hormone replacement therapy (HRT) has been reported to reduce the risk of gastric cancer in population with higher HRT use[26]. We were unable to adjust for this factor in the current study because of the lack of available data. HRT use is low in Taiwan compared with Western countries[27]; therefore, the confounding effect resulting from this factor should be small, if it exists at all. Furthermore, if the association between this potential confounding variable and the risk of gastric cancer is not as strong as the one that has been observed for parity, adjustment of this variable will not qualitatively change the conclusion.

Cigarette smoking[2] and a family history of gastric cancer[28] have been documented as risk factors for gastric cancer in Taiwan. Unfortunately, there is no information available on these variables for the individual study subjects and, thus, they could not be adjusted in the analysis. However, there is no reason to believe that there would be any correlation between these two variables and parity.

An increased susceptibility to infection by *H. pylori* during pregnancy might affect the increased risk of gastric cancer[29]. We could not adjust for this variable because of the lack of information on *H. pylori* infection. However, it has been reported that most women acquired the infection in childhood rather than during pregnancy[30]. Furthermore, there is no reason to believe that *H. pylori* status would be associated with parity, and, therefore, the estimated effect of parity is likely to be free of a confounding effect of *H. pylori* status.

The birth registration system in Taiwan covers only live births and did not include stillbirths and abortions. Therefore we were unable to examine the possible role of gravidity on the risk of gastric cancer. Our study design only allowed for the study of mortality among parous women. Again, we were unable to examine the possible role of nulliparity on the risk of gastric cancer because the birth registry ascertained births rather than pregnancies. The generalizability of this findings is thus limited. Misclassification of menopausal status may have occurred by using age 50 as the cut-off point. Any effect of this is, however, is probably nondifferential (the misclassification is unlikely to be related to parity) and would probably lead to underestimation of the results.

In summary, we found that there was a trend for increasing parity to be associated with increasing risk for gastric cancer among a cohort of young parous women. This study suggests that the relation between parity and risk of gastric cancer should be considered separately for pre- and postmenopausal women.

COMMENTS

Background

Previous studies that examined this association rarely categorized the gastric cancer cases into pre- or postmenopausal because of the lack of sufficient premenopausal gastric cancer cases to support a complete analysis.

Research frontiers

This study was undertaken to examine the association of parity and gastric cancer (the cases are almost all premenopausal women) risk in a cohort of young parous women.

Innovations and breakthroughs

The results of this study suggest that higher parity may increase the risk of death from gastric cancer among premenopausal women.

Applications

This study suggests that the relation between parity and risk of gastric cancer should be considered separately for pre- and postmenopausal women.

Peer review

The study is worthy of being accepted. The study population is large enough to detect minor differences.

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BRIEF ARTICLE

An end-to-end anastomosis model of guinea pig bile duct: A 6-mo observation

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Abstract

AIM: To establish the end-to-end anastomosis (EEA) model of guinea pig bile duct and evaluate the healing process of bile duct.

METHODS: Thirty-two male guinea pigs were randomly divided into control group, 2-, 3-, and 6-mo groups after establishment of EEA model. Histological, immunohistochemical and serologic tests as well as measurement of bile contents were performed. The bile duct diameter and the diameter ratio (DR) were measured to assess the formation of relative stricture.

RESULTS: Acute and chronic inflammatory reactions occurred throughout the healing process of bile duct. Serology test and bile content measurement showed no formation of persistent stricture in 6-mo group. The DR revealed a transient formation of relative stricture in 2-mo group in comparation to control group (2.94 ± 0.17 *vs* 1.89 ± 0.27, P = 0.004). However, this relative stricture was released in 6-mo group (2.14 ± 0.18, P = 0.440).

CONCLUSION: A simple and reliable EEA model of guinea pig bile duct can be established with a good reproducibility and a satisfactory survival rate.

Key words: Animal model; Guinea pig; Anastomosis; Common bile duct; Wound healing

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INTRODUCTION

Bile duct injury (BDI) is a severe consequence of gastrointestinal surgery. Unrecognized or improperly treated biliary injuries can lead to severe complications such as biliary cirrhosis, hepatic failure, and death[1,2]. Treatment of BDI remains a challenge for gastrointestinal surgeons. Reconstruction of bile ducts following iatrogenic injuries is associated with a high risk of stricture and stricture recurrence in the anastomosis[3,4]. Therefore, effective and safe bile duct reconstruction is very important.

Although the method of biliary tract reconstruction has been extensively studied, no consensus is reached concerning the ideal model of biliary tract reconstruction. The most frequently recommended procedure is Roux-Y hepaticojejunostomy (HJ) for its reconstruction[3-5].

However, Roux-Y HJ has its obvious drawbacks, including a large number of postoperative complications, such as a high occurrence of biliary tract stenosis leading to secondary biliary cirrhosis. The diagnostic and thera-

peutic endoscopic access to the biliary tract becomes impaired or hindered since the reconstruction of biliary tract with Roux-en-Y HJ is not anatomical[6]. The changed bile flow pathway is also a cause of disturbance in fat metabolism[7]. In case of poor drainage of the excluded loop, especially when applied in thin biliary tract with an intense inflammatory process, ascending cholangitis may also ensue. As the reconstruction of biliary tract with Roux-en-Y HJ is not physiological, the bile bypass induces gastric hypersecretion leading to a pH change secondary to altered bile synthesis and release of gastrin, therefore peptic ulcer occurs frequently in the long term[8,9].

End-to-end anastomosis (EEA) of bile duct is seldom used in surgical treatment of BDI. However, this procedure is routinely performed during hepatic transplantation with good results[10,11] and can achieve a better long-term outcome than Roux-en-Y HJ. Establishing a physiological bile pathway allows proper digestion and absorption. Also, control endoscopic examination in these patients is possible. Therefore, some authors recommend EEA as the first choice of bile duct reconstruction[6,12].

However, no large-scale clinical trial and a suitable animal model of EEA are available to evaluate the bile duct healing process. Therefore, to gain a better understanding of the healing process after EEA, and provide some valuable information for the etiology, development and prophylaxis of BDI, an animal model of bile duct reconstruction with EEA was established after total resection of common bile duct (CBD) in guinea pigs in this study. Guinea pigs were raised for 2, 3, and 6 mo after operation to observe the short- and long-term healing and possible complications. General conditions, survival rate and histological characteristics of the animals were detected and serology test was performed, as well as content and size of bile duct were measured before and after operation.

MATERIALS AND METHODS

Animal model and experimental design

Thirty-two male guinea pigs weighing 350-400g, purchased from Laboratory of Experimental Animals, Peking University Health Science Center, Beijing, were housed under controlled conditions at a temperature of 21 ± 2℃ and a relative humidity of 30%-70% in a 12-h dark and light cycle. The animals were fasted with free access to water 8 h before and after operation. This study was performed in accordance with the rules for the protections of animals and approved by the Animal Ethical and Welfare Committee of Peking University Health Science Center (LA2008-021).

The animals were randomly divided into control group (group 1), and 2-, 3-, 6-mo groups (groups 2-4) after EEA model was established, 8 in each group.

Microsurgical reconstruction of bile duct

Surgical microscope (XTS-4A Jiangsu Surgical Instruments Company, China), microsurgical instruments (SSW-4, Shanghai Surgical Instruments Company, China), and 10-0 monofilament sutures (Double Arrow, China) were used.

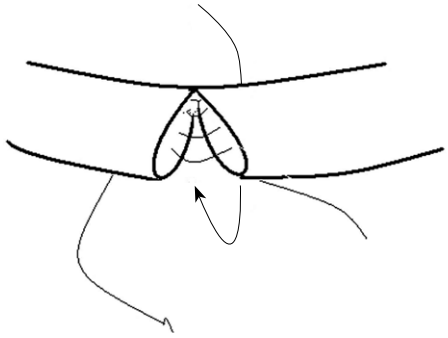

Figure 1 Microsurgical reconstruction of common bile duct with end-to-end anastomosis.

The animals were anesthetized with pentobarbital (30 mg/kg) by intraperitoneal injection under sterile conditions. Peritoneal cavity was accessed *via* a midline incision (approximately 3 cm). After the liver lobes were pressed to the upper region and the duodenum was tracked toward left, the CBD was identified. Gallbladder was drained through a cystic duct joined with hepatic duct into the CBD. The CBD in control group was exposed and freed with forceps. A complete transection between the portal hilus and duodenum was performed with sharp dissection in the other 3 groups. The bile duct was reconstructed with the microsurgical instruments.

EEA was performed with interrupted sutures passing through the layers of the duct wall. The first stitch was placed at the side wall to join the two cut ends. Then, posterior walls of the proximal and distal cut ends were clockwise sutured. After the posterior wall was sutured, anterior wall was sutured in the same way (Figure 1). The proximal and distal cut ends were connected precisely and all stitches were distributed evenly with no tension on the approximate distal and proximal ends. Leaked bile was constantly wiped out to keep the operation area clean. The peritoneal cavity was flushed with normal saline to wash away the remaining bile after suturing was completed. The whole process took about 40 min with 8-10 sutures. No closer was used to disturb the blood supply and bile flow during operation. No T-tube or drainage was placed.

General conditions and survival rate of animals

The general conditions and body weights of guinea pigs were carefully observed. The causes of death of guinea pigs were examined by autopsy. Overall survival rates of the animals were calculated and recorded.

Measurement of bile duct diameter

By the end of two, three and six months after the EEA model was established, guinea pigs in the 4 groups were sacrificed by exsanguination. The maximum diameter of the proximal end (MDP) of CBD and the maximum diameter of anastomosis (MDA) were measured using a sliding caliper. The diameter ratio (DR, DR = MDP/MDA) in every group was calculated to assess the formation of relative stricture. Tissue samples were harvested from the anastomosed bile duct for histological examination.

Histological and immunohistochemical examination

Tissue samples, taken from the anastomosed bile duct, were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm thick cross-sections which were stained with hematoxylin and eosin (HE). Immunohistochemical staining was performed using proliferating cell nuclear antigen (PCNA monoclonal antibody, Medical and Biological Laboratories, Japan). Histological characteristics were reported by the professional pathologist.

Cells were considered positive when their nuclei were stained distinctly brown. Negative control sections were fixed with a safe buffer and positive control sections were used.

The sections of PCNA stained with immunohistochemistry were examined under a light microscope and graded using a modified 0-4 numerical scale provided by Hunt *et al*[13] (Table 1).

Serology examination

Serum alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) levels were measured and analyzed with an automatic biochemical analyzer (Type 5421-04, MISHIMA OLYMPUS CO., Shizuoka-ken, Japan).

Measurement of bile content

The changes of bile contents in 3- and 6-mo groups were analyzed, and the presence of biliary sludge and gallstones was detected. The bile was drained in sterile conditions to examine its pH value. The levels of total bilirubin (TBIL), total bile acid (TBA), and calcium ions were measured with an automatic biochemical analyzer (Type 5421-04, Mishima Olympus Co., Shizuoka-ken, Japan).

Statistical analysis

Levene's test for equality of variances was performed to assess the equality between groups. Independent sample *t* test and Mann-Whiney *U* test were used to compare the differences in the 4 groups. Data were expressed as mean ± SE. Statistical analysis was conducted using the SPSS for Windows (Chicago, Illinois, USA). $P < 0.01$ was considered statistically significant.

RESULTS

General conditions and survival rate of animals

The animals in 4 groups survived throughout the experiment with a survival rate of 97%. One guinea pig in the control group died of anaesthetic intolerance before surgery. The total survival rate was 91% and two guinea pigs (one in 3-mo group and 1 in 6-mo group) died of bile leakage within seven days after operation. No fever, bile leakage, jaundice, infection, cholangitis, peritonitis, or other postoperative complications were noted in the surviving animals during the follow-up. All animals fed with usual diet gained their weight gradually (Table 2) and remained in a good condition till euthanized.

Measurement of bile duct

The mean value of MDA in groups 2-4 was 3.51 ± 0.12 mm, 3.15 ± 0.23 mm, and 3.47 ± 0.16 mm, respectively, which was significantly higher than that (1.71 ± 0.17 mm) in control group ($P < 0.01$). The mean value of MDP in groups 2-4 was 10.24 ± 0.48 mm, 8.66 ± 0.47 mm, and 8.05 ± 0.52 mm, respectively, which was also significantly higher than that (2.95 ± 0.15 mm) of control group ($P < 0.01$). The MDP value in group 2 was the highest. The MDP values were comparable between groups 3 and 4.

The DR indicates the formation of relative stricture and the postoperative DR suggests the remolding status and restoration of physiological function. A significant difference was observed in DR between the control and 2-mo groups (1.89 ± 0.27 *vs* 2.94 ± 0.17, $P = 0.004$). The DR in groups 3 and 4 was 3.24 ± 0.65 and 2.14 ± 0.18, respectively (Table 3).

Table 1 Immunohistochemical grading scale

0	No evidence
1	Occasional evidence
2	Light scattering
3	Abundant evidence
4	Prominent distribution

Modified from[13] for the immunohistochemical grading of proliferating cell nuclear antigen.

Table 2 Body weight of guinea pigs

Groups	*n*	mean ± SE (g)	*P*
Group 1	7	380.86 ± 3.04	
Group 2	8	871.38 ± 15.23	0.002[1,b]
Group 3	7	963.00 ± 39.90	0.002[1,b]
Group 4	7	1101.71 ± 31.96	0.002[1,b]

[1]Mann-Whitney *U* test; [b]$P < 0.01$ *vs* group 1.

Histological examination

Gross inspection of the bile duct after operation revealed that the anastomosed bile duct was narrowed due to local inflammation and edema. The thickened bile duct wall looked like a "stricture ring" (Figure 2).

In this study, normal bile duct contained abundant elastic fibers with a good contract property. Its intima was lined with a single glandular epithelium. Lamina propria contained a small amount of gland elements. Scattered smooth muscle cells were distributed unevenly in the media wall. Adventitia contained fibroblasts and other connective tissues (Figure 3A).

Two months after operation, significant epithelial proliferation, intra- and extramural glandular hyperplasia, fibrous thickening of the duct and dense infiltration of inflammatory cells were noted in the anastomosis (Figure 3B). Inflammatory reactions were gradually subsidized in 3- and 6-mo groups. Hyperplasia of gland elements was observable in 6-mo group (Figure 3C and D).

Immunohistochemical examination

PCNA, a 36-kDa nuclear protein, is an auxiliary protein for DNA polymerase -delta. PCNA expression and distribution

Table 3 Measurement of common bile duct in guinea pigs

Groups	*n*	MDA (mm)		MDP (mm)		DR	
		mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*
Group 1	7	1.71 ± 0.17		2.95 ± 0.15		1.89 ± 0.27	
Group 2	8	3.51 ± 0.12	0.000[1,b]	10.24 ± 0.48	0.001[2]	2.94 ± 0.17	0.004[1,b]
Group 3	7	3.15 ± 0.23	0.000[1,b]	8.66 ± 0.47	0.002[2]	3.24 ± 0.65	0.079[1]
Group 4	7	3.47 ± 0.16	0.000[1,b]	8.50 ± 0.52	0.002[2]	2.14 ± 0.18	0.440[2]

[1]Student's *t* test; [2]Mann-Whitney *U* test; [b]$P < 0.01$ *vs* group 1. MDA: Maximum diameter of anastomosis; MDP: Maximum diameter of the proximal end; DR: Diameter ratio.

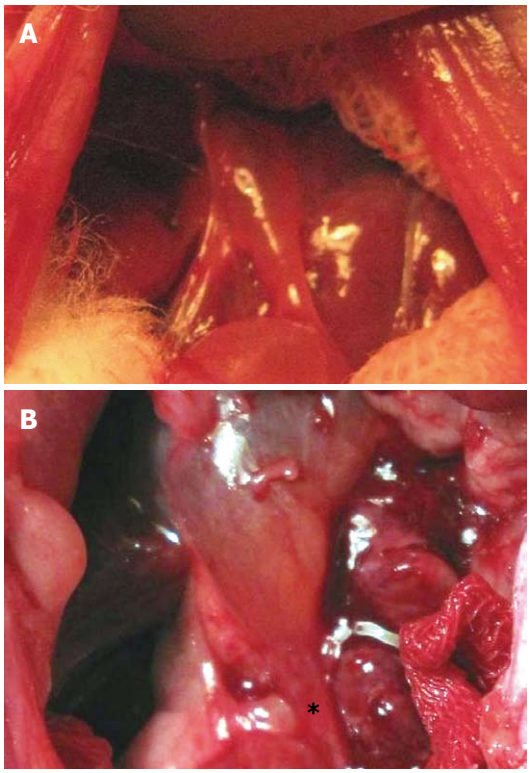

Figure 2 Gross observation of normal common bile duct (A) and its diameter (B) 2 mo after end-to-end anastomosis. Asterisk indicates anastomosed region in 2-mo group.

are correlated with cell proliferation, DNA synthesis, and cell proliferative activity[14,15]. In this study, positive PCNA cells were concentrated on the glandular-epithelia and peribiliary glands of control group. After bile duct reconstruction in 2- and 3-mo groups, an increased number of positive cells were distributed in all layers of the duct wall. By the end of six months, positive PCNA cells were located mainly in glandular elements and epithelial cells (Figure 4). The number of proliferated PCNA cells was significantly greater in groups 2-3 than in control group ($P < 0.01$) (Table 4).

Serum ALP and GGT levels

One serum sample taken from control group was abandoned due to contamination. The mean serum ALP and GGT level was 55.33 ± 8.44 U/L and 14.67 ± 2.76 U/L, respectively, in control group and groups 2-4 ($P < 0.01$, Table 5).

Table 4 Semi-quantitative analysis of proliferating cell nuclear antigen expression in bile duct of guinea pigs

Groups	*n*	mean ± SE	*t*	*P*
Group 1	7	1.57 ± 0.535		
Group 2	8	3.63 ± 0.518	-7.511	0.000[b]
Group 3	7	3.14 ± 0.378	-6.351	0.000[b]
Group 4	7	1.71 ± 0.488	-0.522	0.611[b]

[b]$P < 0.01$ *vs* group 1.

Table 5 Serum alkaline phosphatase and γ-glutamyltransferase levels in guinea pig (U/L)

Groups	*n*	ALP (U/L)		GGT (U/L)	
		mean ± SE	*P*	mean ± SE	*P*
Group 1	6	53.33 ± 8.44		14.67 ± 2.76	
Group 2	8	76.63 ± 9.95	0.145[1]	13.00 ± 1.04	0.560[2]
Group 3	7	63.29 ± 5.18	0.424[1]	14.00 ± 1.50	0.829[1]
Group 4	7	62.00 ± 13.22	0.691[1]	16.43 ± 1.04	0.774[2]

[1]Student's *t* test; [2]Mann-Whitney *U* test. ALP: Alkaline phosphatase; GGT: γ-glutamyltransferase.

Measurement of bile contents

The bile contents were measured to show whether there is a tendency to form gallstones. No biliary sludge or gallstones were found in the biliary system of all groups, and no significant difference was observed in levels of TBIL, TBA, calcium ions and pH value among the 4 groups (Table 6).

DISCUSSION

In this study, EEA was performed instantly after total transection of CBD in guinea pigs. A few weeks after EEA, the anastomosed bile duct was narrowed due to local inflammation, edema and proliferation of glandular elements. The thickened bile duct wall looked and functioned as a "stricture ring", leading to a significantly higher hydrodynamic pressure on the proximal bile duct end and noticeable dilation of the proximal bile duct end above the stenosis zone. The increased pressure of the proximal bile duct end would pass on the pressure to the anastomosed area of bile duct and influence its remolding. The "stricture ring" took on adaptive changes with inflammation gradually subsidized after a

Table 6 Bile contents in guinea pigs

Groups	*n*	TBIL (μmol/L)		TBA (μmol/L)		Ca^{2+} (mmol/L)		pH	
		mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*
Group 1	7	32.44 ± 3.43		11668.38 ± 1549.42		0.78 ± 0.06		8.87 ± 0.07	
Group 3	7	28.5 ± 7.92	0.622[1]	15981.67 ± 2295.02	0.136[1]	1.17 ± 0.13	0.014[1]	8.86 ± 0.08	0.908[1]
Group 4	7	39.19 ± 9.97	0.565[2]	18260.25 ± 4164.40	0.164[1]	1.24 ± 0.17	0.064[2]	8.82 ± 0.07	0.642[1]

[1]Student's *t* test; [2]Mann-Whitney *U* test. TBIL: Total bilirubin; TBA: Total bile acid.

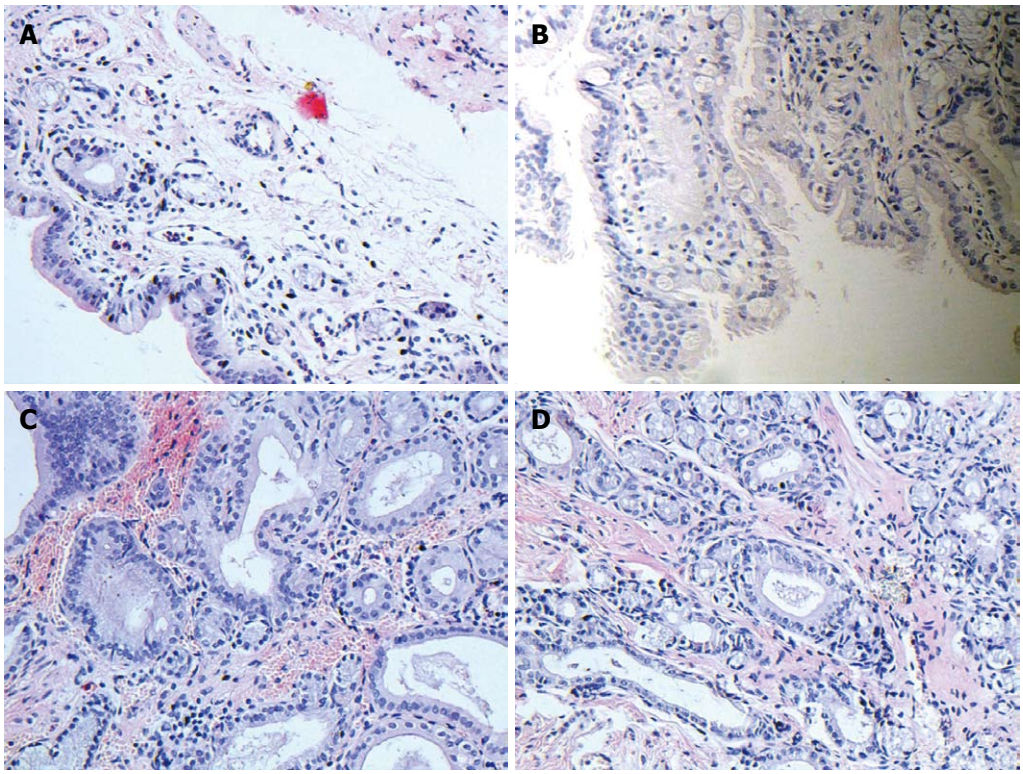

Figure 3 Histological examination of bile duct in all groups (original magnification, × 200). A: Normal bile duct intima; B: Gland proliferation and infiltration of inflammatory cells in 2-mo group; C: Fibrous thickening of bile duct and dense infiltration of inflammatory cells in 3-mo group; D: Epithelial proliferation as well as intra- and extramural glandular hyperplasia in bile duct.

few months. The MDA and MDP values were significantly higher in groups 2-4 than in control group. Therefore, a single MDA parameter could not reliably reflect the remolding process of the anastomosed bile duct. Moreover, the body weight of guinea pigs increased throughout the experiment in 6-mo group. The DR parameter of (DR = MDP/MDA) was used to assess the remolding status and evaluate whether there is a relative stricture formation. If the DR was significantly higher in groups 2-4 than in control group, a relative stricture would form.

The DR was significantly higher in 2-mo group than in control group, which might be the indication for relative stricture formation. The animals remained in a good condition with their weight gradually increased. The MDP, MDA and DR values were lower in 6-mo group than in 2-mo group, while comparable to those in 3-mo group. No significant difference was noted in levels of ALP, GGT, and bile contents among 4 groups, indicating that normal bile duct anatomy and physiological function can be gradually restored after a transient formation of "relative stricture".

Although the body weight of guinea pigs was notably increased in six months, the bile duct parameters did not increase in proportion, suggesting that the growth of animals is not the only cause of bile duct enlargement. We hypothesize that besides hydrodynamic pressure of bile flow to the side walls and normal growth of the animals, neurological factors may also account for the changes during bile duct remolding.

Consequently, the postoperative remolding process in 6-mo group was a synergetic and balanced result of tissue injury and repair, as well as hydrodynamic and neurological changes in injured CBD. Further study is needed to explain the neurological mechanism underlying the postoperative remolding process of bile duct.

It has been shown that biliary stasis can induce pigment stone formation in animals[16,17] and in patients with biliary stricture[18]. It was reported that the increased bile

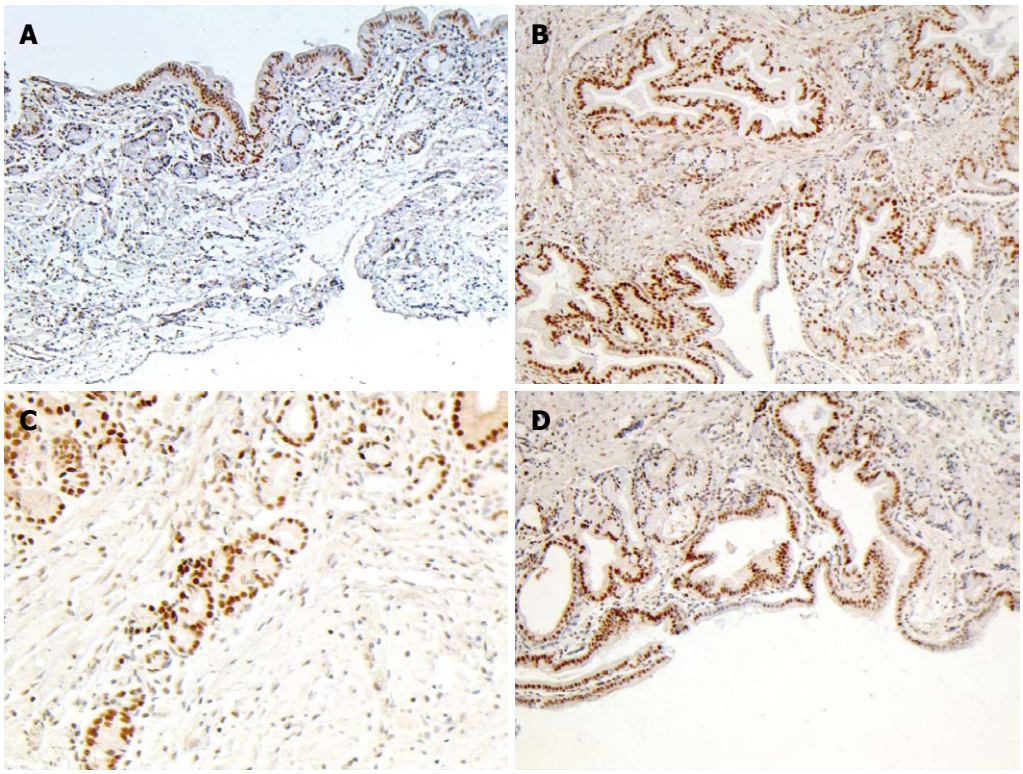

Figure 4 Proliferating cell nuclear antigen immunohistochemical staining. A: Distribution of proliferating cell nuclear antigen (PCNA) positive cells in normal bile duct (original magnification, × 100); B, C: Positive PCNA cells distributed in glandular-epithelia and peribiliary glands in 2- and 3-mo groups, respectively (B: original magnification, × 100; C: original magnification, × 200); D: Positive PCNA cells located mainly in glandular elements and epithelium cells (original magnification, × 100).

pH and changes in bile contents when ligation results in bile duct stricture are the early events, leading to the formation of gallstone[17,19]. In our experiment, the levels of TBIL, TBA, calcium ions and the pH value were measured. No biliary sludge or gallstones were found in the biliary system of guinea pigs.

Guinea pig is an ideal animal for the reconstruction of bile duct with EEA. The EEA model is frequently used in studies of biliary system, especially in investigation of gallstone formation[17,19]. Rats have no gallbladder and the diameter of their CBD is only 1 mm[20]. The anatomy of bile duct in guinea pigs is very similar to that of human beings. Since the diameter of bile duct in guinea pigs is approximately 1.7 mm, it is easy to reconstruct the bile duct with a good reproducibility. The bile contents differ in species. The bile in guinea pigs contains the same bilirubinic acid as in human beings, while the bile in rabbits contains biliverdinic acid which renders it unusable in this respect[21]. Moreover, although dogs might be the better candidates for the reconstruction of bile duct, guinea pigs were chosen in this preliminary experiment from the animal welfare and ethical point of view.

In the present study, the histological characteristic of normal CBD tissue taken from guinea pigs were similar to those taken from human beings, which are consistent with the reported findings[22-24], indicating that guinea pigs are ideal for the establishment of EEA models.

The tissue repair of injured biliary tract is a scar healing process. Two important changes in tissue repair can restore the morphological consistency and physiological function, namely the formation of granulation tissue with contractile properties and the epithelial cell proliferation, migration and the closure of the wound[25]. MFB is the major constituent of inflammatory and reparative granulation tissues. By forming a net work of contracting system, MFB may last scar contraction and result in stricture formation. MFB disappear due to apoptosis when the epithelialization is completed and the remolding process becomes stable[26,27]. The presence of MFB can lead to excessive scarring and fibrotic conditions. Geng and his colleagues established the bile duct anastomosis model of dogs by making an incision on the anterior wall of CBD with one third of its circumference, and found that the number of myofibroblasts can reach its peak 3 mo after operation, and decrease due to apoptosis 6 mo after operation[28], suggesting that six months is enough for the examination of the healing process of bile duct anastomosis. However, the long-term outcome is critical in surgical treatment of BDI in clinical practice. No biliary anastomosis stricture formation is a proof of successful surgical management. Therefore, further study is needed to observe the longer postoperative outcome.

In this preliminary study, we presented a simple and reliable animal EEA model for bile duct reconstruction with a good reproducibility and satisfactory survival rate. No permanent biliary anastomosis stricture was noticed in 6-mo group and no serology or bile content revealed stricture formation. The overall animal survival rate was 91%. The animals gained their weight with no postoperative biliary obstruction found in all groups.

In conclusion, the EEA animal model of bile duct established in this study can be used in studies of BDI etiology, development, and possible prophylaxis, and provide some valuable information for the postoperative healing process of bile duct.

COMMENTS

Background

Treatment of bile duct injury (BDI) remains a great challenge for gastrointestinal surgeons. No consensus has been reached concerning the ideal method for bile duct reconstruction. No large scale clinical study is available on bile duct reconstruction.

Research frontiers

Some surgeons prefer end-to-end anastomosis (EEA) as a more physiological method in bile duct reconstruction. However, no large-scale clinical study or suitable animal model is available or analyzed. In this study, by establishing a reliable animal EEA model of common bile duct (CBD) and observing the postoperative results of histological, immunohistochemical exanimation, serologic and bile content analysis, as well as bile duct parameters, the authors demonstrated that EEA can be utilized in treatment of BDI.

Innovations and breakthroughs

The authors provided a simple and reliable animal EEA model of CBD with a good outcome in this study, which may shed light on studies of BDI etiology, development, and possible prophylaxis.

Applications

The animal EEA model of CBD we established in the present study can be utilized in studies on BDI etiology, development, and possible prophylaxis as well as provide some valuable information for the post-operative healing process of EEA.

Terminology

EEA, an end-to-end anastomosis procedure, is a more preferable choice of treatment than Roux-en-Y maneuver. MFB are the myofibroblasts with α-SMA expression in stress fibers. In wound healing, inflammation mediators and mechanical tension lead to generation of actin-containing microfilaments or stress fibers which confer contractile property to fibroblasts, and convert them into terminally differentiated MFB. MFB are the major constituent of inflammatory and reparative granulation tissue and can last scar contraction and stricture formation.

Peer review

In this study, the animals gained their weight and no postoperative biliary obstruction was observed in any group after bile duct reconstruction with EEA in this study, showing that EEA is a simple and reliable procedure for bile duct reconstruction with a satisfactory survival rate, which provides some valuable information for the postoperative healing process of bile duct.

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BRIEF ARTICLE

RRAS: A key regulator and an important prognostic biomarker in biliary atresia

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Abstract

AIM: To characterize the differentially expressed gene profiles in livers from biliary atresia (BA) patients including, ascertain genes, functional categories and pathways that play a central role in the pathogenesis of BA, and identify the novel prognostic markers for BA.

METHODS: Liver tissue samples from control patients, neonatal cholestasis patients, and BA patients at the age of < 60 d, 60-90 d, and > 90 d were pooled for DNA microarray analysis. Bioinformatics analysis was performed using, series test cluster of gene ontology, and Pathway-Finder software. Reverse-transcription polymerase chain reaction was performed to confirm changes in selected genes. Relation between RRAS gene expression and prognosis of 40 BA patients was analyzed in a 2-year follow-up study.

RESULTS: The 4 identified significant gene expression profiles could confidently separate BA liver tissue from normal and other diseased liver tissues. The included genes were mainly involved in inflammation response and reconstruction of cellular matrix. The significant pathways associated with BA were primarily involved in autoimmune response, activation of T lymphocytes and its related cytokines. The *RRAS*, *POMC*, *SLC26A6* and *STX3* genes were important regulatory modules in pathogenesis of BA. The expression of RRAS was negatively correlated with the elimination rate of jaundice and positively correlated with the occurrence rate of cholangitis.

CONCLUSION: Autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA. The *RRAS* gene is an important regulatory module in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

Key words: Biliary atresia; DNA microarray; Bioinformatics; RRAS; Prognostic biomarker

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INTRODUCTION

Biliary atresia (BA) is a devastating disease of infants, invariably leading to cirrhosis, end-stage liver disease, and death if untreated[1]. A recent review reported that BA may involve a primary perinatal hepatobiliary viral infection and a secondary autoimmune-mediated bile duct inju-

ry[2]. However, the cause and pathogenesis of BA remain largely unknown.

Microarray technology, emerged as an indispensable research tool for gene expression profiling, has been used to study the mechanism underlying BA, and allows the simultaneous analysis of thousands of transcripts within a single experiment[3]. Some studies have been performed to investigate the gene expression profiling of livers from BA patients[4-6]. However, to our knowledge, none of them was designed to identify genes that play a key role in the pathogenesis and prognosis of BA. In the current study, DNA microarrays for whole genome gene expression and bioinformatics analysis were used to characterize the differentially expressed gene patterns of normal livers and livers from BA patients at different ages, as well as to ascertain the genes and pathways that play a central role in the pathogenesis of BA. Furthermore, reverse-transcription polymerase chain reaction (RT-PCR) was performed to confirm the changes in selected genes. The relation between selected gene expression and prognosis of BA patients was also analyzed.

MATERIALS AND METHODS

Patients and specimens

Biopsy specimens were obtained from livers of 9 patients with BA, 3 patients with neonatal cholestasis and 3 control patients suffering from liver trauma (as normal control) at Children's Hospital of Fudan University from November 2007 to December 2008. Nine patients with BA were further divided into < 60 d group (n = 3), 60-90 d group (n = 3) and > 90 d group (n = 3). Liver samples from 3 groups of BA patients, neonatal cholestasis and control groups were immediately dissolved in a RNAlater RNA stabilization reagent (Qiagen, Germany) and then stored at -80℃. Liver samples from each group were pooled and total RNA was isolated from them for DNA microarray experiments. Clinical data about these patients are summarized in Table 1. Liver samples were collected from the other 14 patients with neonatal cholestasis and 40 patients with BA for RT-PCR experiments. All subjects gave their informed consent to participate in the study which was approved by the Research Ethics Committee of Fudan University.

RNA extraction, processing and microarray analysis

Total RNA was extracted from liver tissue samples using the Trizol reagent (Invitrogen) according to its manufacturer's protocol, and then further purified using a NucleoSpin RNA clean-up kit (Macherey-Nagel, Germany). Quantification analysis of RNA was performed on a spectrophotometer and quality of RNA was analyzed by denaturing formaldehyde gel electrophoresis. Five micrograms of total RNA from each group was amplified and labeled with biotin using an Illumina total Prep RNA amp kit (Ambion, Austin,TX, USA) and hybridized to Illumina's Sentrix Human-6 (Version 3) Expression BeadChips containing 48000 transcripts (Illumina, San Diego, CA, USA). Three duplicated chips were also used in each group to test the variations in duplications from the same pooling. The hybridized Illumina chips were scanned on a BeadArray reader (Illumina, San Diego, CA, USA) and microarray analysis was performed using the BeadStudio software (Illumina, San Diego, CA, USA). Raw data were normalized using the cubic spline method and the resulting genes were filtered. Finally, only genes with a differential expression score (Diffscore) greater than 20 or less than -20 were included.

Bioinformatics analysis of differentially expressed genes

Differentially expressed genes were analyzed by a series test of cluster (STC) to search a set of model expression profiles that were distinct in 5 groups as previously described[7,8]. These profiles were assigned to significant gene ontology categories using series test cluster of gene ontology (STC-GO)[9], and analyzed with the Pathway-Finder software to obtain the significance of pathway categories[10,11]. Moreover, dynamic gene networks were constructed to find the key genes that may play a central role in the pathogenesis of BA[12,13]. The principle and algorithmic details are available in supplementary data.

Validation of microarray data by RT-PCR

RT-PCR was performed on liver tissue samples from 14 patients with neonatal cholestasis and 40 patients with BA to confirm changes in selected genes, including RRAS, POMC, SLC26A6 and STX3. Total RNA was extracted from liver tissue samples and purified as previously described[14]. Five micrograms of total RNA was reverse transcribed using MMLV reverse transcriptase (Merck, Germany) and random primers in a 20 μL reaction volume at 42℃ for 1 h. Oligonucleotide primers for the RRAS, POMC, SLC26A6, STX3 and β-actin are shown in Table 2. The PCR conditions were as follows: an initial denaturation at 94℃ for 5 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing at 58℃ for 30 s, extension at 72℃ for 30 s, and a final extension at 72℃ for 7 min. The PCR products were separated by electrophoresis and UV illuminated on a 2% agarose gel containing ethidium bromide (0.5 μg/mL). The gel image was stored using the UVP gel documentation system 5000 (Ultra-Violet Products Ltd., Cambridge, United Kingdom). Expression levels of the selected genes relative to β-actin were measured with densitometric scanning using the multi-analysis/PC system (Bio-Rad, Hercules, California, USA).

Prognostic biomarker and follow-up research

Fibrosis of liver biopsy specimens from 40 patients with BA was histologically classified into different groups. Elimination rate of jaundice (TB < 20 μmol/L) within 6 mo after operation, 2-year survival rates of cholangitis patients and of 40 BA patients were calculated. The diagnostic criteria for cholangitis included fever, increasing jaundice, acholic stools, with other causes of infection excluded. Follow-up data were obtained from our outpatient and inpatient referrals, as well as from interview by

Table 1 Microarray analysis showing clinical characteristics of biliary atresia patients

Case No.	Gender	Age	TB/DB	ALT/AST	AKP/GGT	Albumin	Disease	Group
1	Female	50 d	171/130	132/254	604/609	35.8	BA	1
2	Male	49 d	291/231	148/155	581/408	39.4	BA	1
3	Male	57 d	148/117	189/166	493/535	35.2	BA	1
4	Female	73 d	145/118	111/153	460/1460	36.3	BA	2
5	Male	84 d	160/127	100/149	713/1278	37.3	BA	2
6	Female	66 d	129/108	121/165	632/1235	39.2	BA	2
7	Female	103 d	151/112	85/62	451/360	39.4	BA	3
8	Female	97 d	118/89	74/78	522/501	34.3	BA	3
9	Male	110 d	155/121	105/97	377/912	35.4	BA	3
10	Female	77 d	102/89	201/137	234/317	39.4	Cholestasis	4
11	Male	64 d	137/108	404/267	584/1044	37.2	Cholestasis	4
12	Female	55 d	144/112	389/266	612/1339	41.0	Cholestasis	4
13	Male	4 yr	16/9	33/35	200/50	39.4	Liver trauma	5
14	Male	6 yr	10/4	20/25	192/44	40.1	Liver trauma	5
15	Male	4 yr	12/4.7	29/37	101/38	37.1	Liver trauma	5

TB: Total bilirubin; DB: Direct bilirubin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AKP: Alkaline phosphatase; GGT: γ-glutamyl transferase; BA: Biliary atresia.

Table 2 Sequences of primers in selected genes used in reverse-transcription polymerase chain reaction

Gene name	Primer sequences
RRAS	F: TTGGTCGGGAACAAGGCAGAT
	R: CTCGTCCACGTTGAGACGCAGT
POMC	F: GAGAGCAGCCAGTGTCAGG
	R: GAAGTGGCCCATGACGTACT
SLC26A6	F: CGGTATCCTGTGCGTGACT
	R: GGAAGTGCCAAACAGGAAGT
STX3	F: GGCAAAAAGACAACCGATGA
	R: TGTCGTGAAGCTCCTTGATG
β-actin	F: GGGAAATCGTGCGTGCATT
	R: CAGGCAGCTCGTAGCTCTT

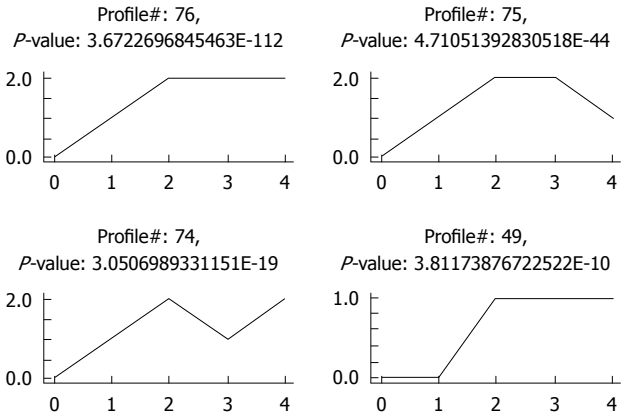

Figure 1 Four most significant expression profiles in liver samples from biliary atresia patients. 0: Normal control group; 1: Neonatal cholestasis group; 2: Group of biliary atresia (BA) patients > 90 d; 3: Group of BA patients at the age of 60-90 d; 4: Group of BA patients < 60 d. Y axis represents the expression change expressed as log2 [v(i)/v(0)].

telephone or questionnaires. These patients were further classified based on the follow-up data, including presence of jaundice 6 mo after operation, occurrence of cholangitis within 2 years after operation, and 2-year survival rate.

Statistical analysis

Data were expressed as mean ± SD. Variations in duplications were detected by Fisher's exact test, χ^2 test, *t*-test and Cochran-Mantel-Haenszel test using the STATA 8.0 software (Stata Co., College Station, TX, USA). Pair-wise test was used to confirm the limited variations in duplications from the same pooling. $P < 0.05$ was considered statistically significant.

RESULTS

Differentially expressed gene profiles in liver tissue samples from BA patients

Total RNA was extracted from liver tissues of the normal group, neonatal cholestasis group, and 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d). Denaturing formaldehyde gel electrophoresis showed no degradation (data not shown). Illumina's Sentrix Human-6 (Version 3) Expression BeadChip was used for each of pooled liver tissue samples from the 5 groups. Pair-wise test confirmed a limited variation in duplications from the same pooling (data not shown). A total of 795 differentially expressed genes were identified from different groups with a Diffscore greater than 20 or less than -20. Further STC analysis yielded 80 expression profiles. Of these expression profiles, 20 were statistically significant ($P < 0.05$) and 4 had the lowest *P* value (Figure 1). These 4 expression profiles could confidently separate livers in groups of BA patients from those in normal and neonatal cholestasis groups. Specifically, all the differentially expressed genes in 5 groups were included in profile 76, which showed no significant difference in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d). The 611 genes represented in profile 75 were mainly involved in inflammation mediated by activation of T lymphocytes, and reconstruction of extracellular matrix. As shown in Figure 1, the global expression level of

Table 3 Significant pathways involved in pathogenesis of biliary atresia

Pathway name	*P* value	Profile No.
Cell adhesion molecules	0.000118	Profile49
Regulation of actin cytoskeleton	0.000739	Profile49
T Leukocyte transendothelial migration	0.001738	Profile49
Asthma	0.002023	Profile49
Allograft rejection	0.003243	Profile49
Systemic lupus erythematosus	2.44E-05	Profile74
Lysosome	0.0002109	Profile74
NF-kappa B signaling pathway	0.0036605	Profile74
MAPK signaling pathway	0.0052322	Profile74
Allograft rejection	0.0058515	Profile74
Graft-versus-host disease	0.0071277	Profile74
Type I diabetes mellitus	0.00781	Profile74
Chemokine signaling pathway	1.81E-08	Profile75
Matrix_Metalloproteinases	6.52E-07	Profile75
Cytokine-cytokine receptor interaction	3.59E-05	Profile75
T cell receptor signaling pathway	4.34E-05	Profile75
Antigen processing and presentation	0.000337	Profile75
Leukocyte transendothelial migration	0.0010211	Profile75
Lysosome	2.09E-07	Profile76
Toll-like receptor signaling pathway	0.000284	Profile76
T cell receptor signaling pathway	0.000388	Profile76
Chemokine signaling pathway	0.000755	Profile76
Asthma	0.000841	Profile76
Matrix_Metalloproteinases	0.001402	Profile76
Allograft rejection	0.001697	Profile76

the genes in profile 75 was much lower in livers from the group of BA patients at the age of < 60 d than from the groups of BA patients at the age of 60-90 d and > 90 d. The 372 genes represented in profile 74 were associated with an apoptotic pathway and inflammatory response mediated by nuclear factor-κB (NF-κB). The global expression level of profile 74 genes was much lower in the group of BA patients at the age of 60-90 d than in the groups of BA patients at the age of < 60 d and > 90 d. Moreover, the 285 genes represented in profile 49 were mainly involved in inflammatory response mediated by the major histocompatibility complex (MHC) class II antigen. The global expression level of profile 49 genes was much higher in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d) than in the normal and neonatal cholestasis groups. Little variance in gene expression was observed neither in the 3 groups of BA patients at different ages (< 60 d, 60-90 d and > 90 d) nor in the normal and neonatal cholestasis groups (See supplementary data for a complete list of these 4 expression profiles).

Involvement of significant pathways in BA focused on autoimmune response associated with inflammatory response of T lymphocytes

Based on the Kyoto Encyclopedia of Genes and Genomes Database and the most significant 4 gene expression profiles, Fisher's exact test and χ^2 test were performed to identify the significant pathways involved in BA as previously described[15]. The significant pathways ($P < 0.01$) highly associated with BA were mainly focused on (1) autoimmune response associated with asthma, systemic lupus erythematosus, allograft rejection graft-versus-host disease, type I diabetes mellitus, antigen processing and presentation; (2) activation of T lymphocytes and inflammatory response including transendothelial migration of T leukocytes, cell adhesion molecules, NF-κB and MAP kinase (MAPK) signaling pathways, chemokine signaling pathway, cytokine-cytokine receptor interaction, transendothelial migration of leukocytes, Toll-like receptor signaling pathway; and (3) reconstruction of extracellular matrix, including matrix-metalloproteinases. The significant pathways are shown in Table 3.

Table 4 Genes with the highest degree and k-core in dynamic gene networks

Gene symbol	Definition	Degree	k-core
RRAS	Homo sapiens related RAS viral (r-ras) oncogene homolog (RRAS), mRNA	12	6
POMC	Homo sapiens POMC, transcript variant 1, mRNA	12	6
SLC26A6	Homo sapiens SLC26A6, transcript variant 3, mRNA	12	6
STX3	Homo sapiens STX3, mRNA	10	6

Table 5 Reverse-transcription polymerase chain reaction showing relative expression levels of RRAS, POMC, SLC26A6 and STX3 (mean ± SD)

Group	POMC	SLC26A6	RRAS	STX3
Biliary atresia (n = 40)	0.58 ± 0.090	0.43 ± 0.054	0.89 ± 0.103	0.61 ± 0.074
Neonatal cholestasis (n = 14)	0.41 ± 0.081	0.30 ± 0.029	0.47 ± 0.074	0.51 ± 0.045
P-value	0.031	0.023	0.004	0.017

Construction of dynamic gene networks

Dynamic gene networks[16] were constructed to find the key regulators that may play a central role in the pathogenesis of BA (Figure 2). Circles indicate genes in the 4 expression profiles, solid lines indicate direct interactions, size of circles indicates their interactions with other molecules, coloring is classified according to the k core and red indicates high k core. The genes with the highest degree and k-core from Figure 2, are listed in Table 4, including the related RAS viral (r-ras) oncogene homolog (RRAS), POMC, SLC26A6 and STX3 genes, indicating that they play a crucial role in the pathogenesis of BA.

Expression of RRAS, POMC, SLC26A6 and STX3 genes in liver tissue samples from patients with BA confirmed by RT-PCR

The mRNA expression levels of RRAS, POMC, SLC26A6 and STX3 in liver tissue samples from 40 patients with BA and 14 patients with neonatal cholestasis, measured in order to validate the results derived from microarray data, were significantly higher in liver tissue samples from patients with BA than from those of neonatal patients

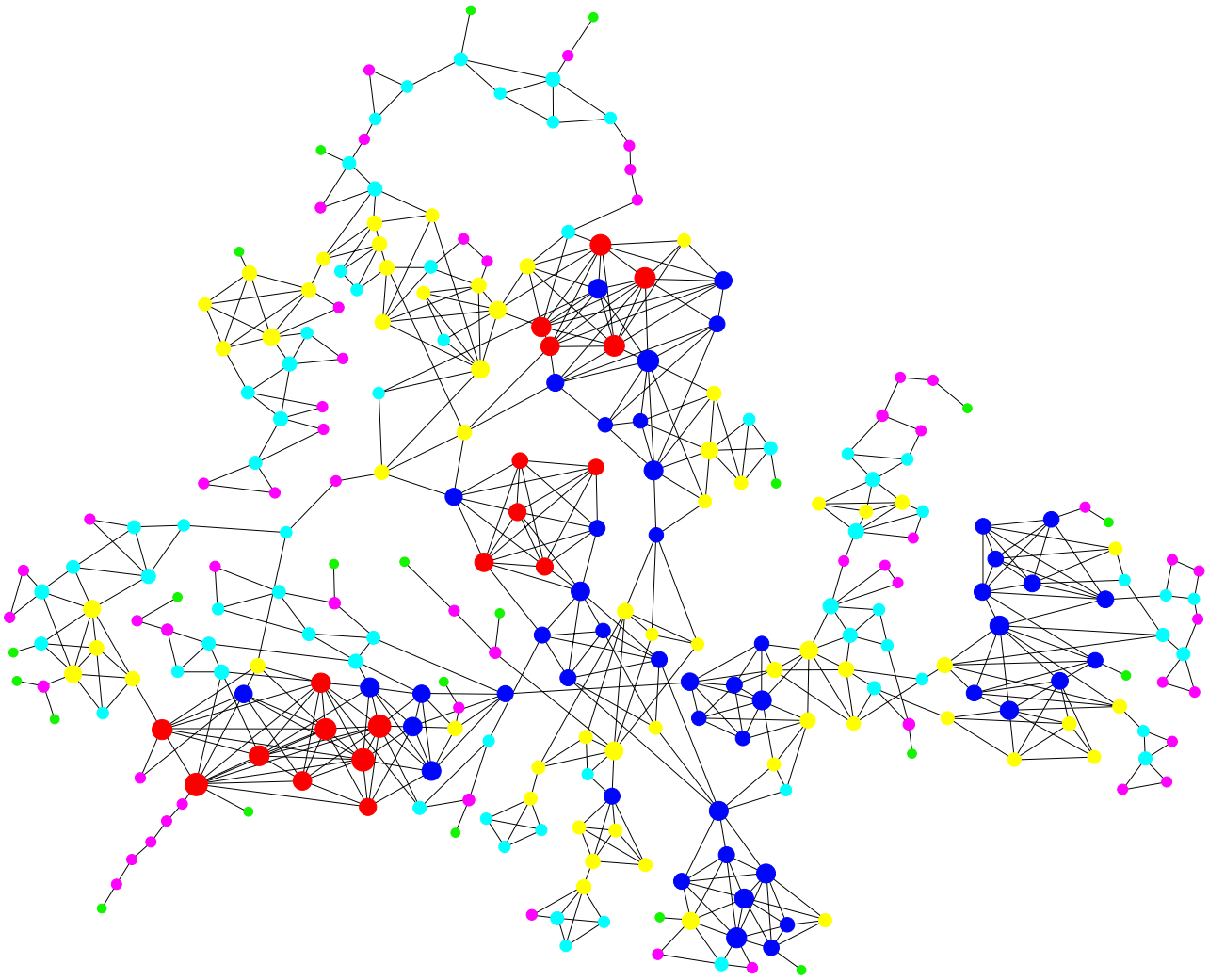

Figure 2 Dynamic gene networks constructed showing the key regulators that may play a central role in the pathogenesis of biliary atresia. Circles indicate genes in the 4 expression profiles, solid lines indicate direct interactions, and size of circles indicates their interactions with other molecules. Coloring is classified according to the k core, and red indicates high k core.

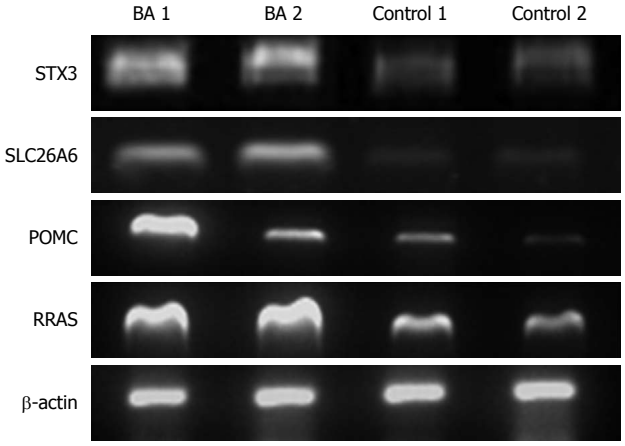

Figure 3 Reverse-transcription polymerase chain reaction showing expression levels of RRAS, POMC, SLC26A6, and STX3 genes. BA: Biliary atresia; Control: Neonatal cholestasis.

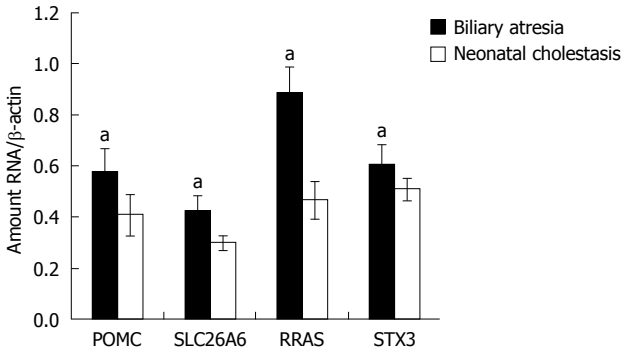

Figure 4 Reverse-transcription polymerase chain reaction showing significantly higher relative expression levels of RRAS, POMC, SLC26A6 and STX3 genes in biliary atresia patients (n = 40) than in neonatal cholestasis patients (n = 14). [a]$P < 0.05$ *vs* liver tissue samples from neonatal cholestasis patients.

with cholestasis ($P < 0.05$, Table 5, Figures 3 and 4). The mRNA expression level of the RRAS gene increased 1.9-fold in BA patients ($P < 0.05$).

Correlation between expression of RRAS in liver tissue samples and prognosis of BA patients

To address whether the RRAS expression is associated with the prognosis of BA patients, we performed a 2-year

Table 6 Fibrosis scores for different groups of biliary atresia patients at different ages

Group	Fibrosis score (*n*)					Total
	0	1	2	3	4	
< 60 d	1	3	8	2	0	14
60-90 d	0	2	3	6	4	15
> 90 d	0	0	1	4	6	11
Total	1	5	12	12	10	40

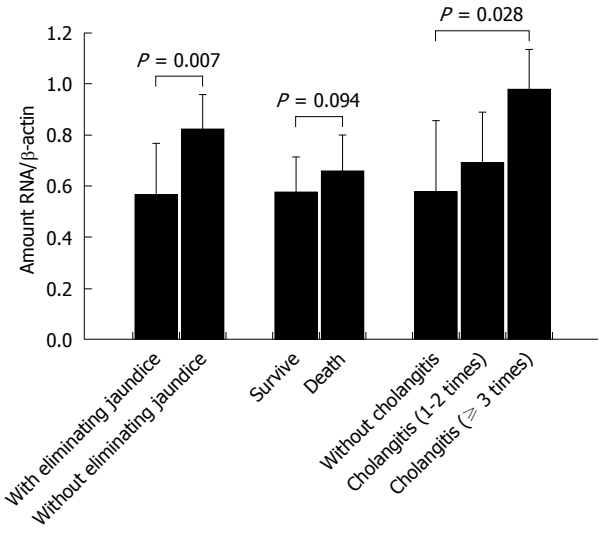

Figure 5 Correlation between expression of RRAS in liver tissue samples and prognosis of biliary atresia patients within 2 years.

follow-up study in 40 patients with BA. Six months after operation, The RRAS expression level was significantly higher in patients with their jaundice not eliminated than in those with their jaundice eliminated (P = 0.007). Furthermore, the expression level of RRAS was significantly higher in patients with cholangitis reoccurred 3 or more times than in those with no cholangitis recurred (P = 0.028). However, no significant difference in RRAS expression was found between the surviving and dead patients (P = 0.094, Figure 5). In addition, the fibrosis was more serious in group of BA patients at the age of > 60 d than in group of BA patients at the age of < 60 d (Table 6), which was consistent with that observed in profile 75.

DISCUSSION

The pathogenesis of BA has not yet been delineated. It has been shown that factors such as genetic susceptibility, congenital heteroplasia, and infectious and abnormal immune response lead to BA[17-23], and its clinical course and surgical outcome are correlated with the age of such patients[24-26]. Although there are some studies involving DNA microarrays in BA[4-6], very few studies are available on gene expression profiling of BA at its different stages of clinical course. That is why the clinicopathologic characteristics of BA vary with the age of such patients.

The differential gene expression patterns of RRAS in liver tissue samples from BA patients at different ages, as well as normal liver tissue samples and liver tissue samples from neonatal cholestasis patients were characterized in this study using the expression DNA microarray technology and bioinformatics. The 4 significant expression profiles identified using STC could confidently separate BA liver tissues from normal and diseased liver tissues. STC-GO analysis revealed that the genes represented in the 4 profiles were mainly involved in inflammatory response and reconstruction of extracellular matrix. Notably, as validated by fibrosis classification, profile 75 showed that the expression level of genes involved in fibrosis and inflammation was much lower in BA patients at the age of < 60 d than in those at the age of > 60 d, which may explain why BA patients at the age of < 60 d often have a good prognosis after a Kasai's operation[27]. Additionally, this phenomenon may also result from fibrosis due to the continuous hepatic inflammatory response-induced activation of stellate cells[28]. Moreover, a set of genes were involved in apoptosis represented in profile 74, which is in agreement with the reported findings[29]. In this profile, the BA patients at the age of > 90 d and < 60 d showed obvious inflammatory response and apoptosis mediated by NF-κB, which might be associated with the inflammatory response of local bile ducts in BA patients at the age of < 60 d and severe inflammatory response induced by fibrosis in BA patients at the age of > 90 d.

Mack *et al*[30,31] showed that $CD4^+$ Th1-mediated bile duct inflammation is responsible for the development of BA. Profile 74 in the present study contains a set of genes associated with MHC class Ⅱ antigen-mediated Th1 inflammatory response, which is in agreement with the findings of Mack *et al*[31] and Osada *et al*[32]. It is well known that antigens associated with MHC class Ⅱ can bind to T cell receptors of $CD4^+$ Th1 cells, and thereby produce functional T lymphocytes. Furthermore, the significant pathways highly associated with BA were mainly focused on the autoimmune response, activation of T lymphocytes and its related cytokines, suggesting that autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA, which is consistent with the widely accepted hypothesis of BA[2,22,23,32].

In this study, the RRAS, POMC, SLC26A6 and STX3 genes were found to be important regulatory modules in BA. The RRAS gene is a component of the MAPK signaling pathway with GTP kinase activity[33,34]. The MAPK pathway is associated with BA[35]. Based on the results of this study, it is reasonable to speculate that the RRAS gene plays an important role in the pathogenesis of BA. The human POMC gene is located on chromosome 2p23.3 encoding a preprohormone. The adrenocorticotropin hormone and α melanocyte-stimulating hormone are cleavage products of POMC, which are associated with immune regulation and participate in the pathogenesis of experimental autoimmune encephalomyelitis[36,37]. The SLC26A6 is an anion exchanger involved in the secretion of bile acid. The STX3 has been implicated in the development and differentiation of dendritic cells[38,39]. Nonetheless,

the precise role of these genes in the pathogenesis of BA needs to be further elucidated.

Given the key role of RRAS gene in the pathogenesis of BA, we evaluated the relation between the expression of RRAS and prognosis of BA patients through a 2-year follow-up study. The RRAS expression was negatively correlated with the elimination rate of jaundice and positively correlated with the occurrence rate of cholangitis, indicating that up-regulation of RRAS expression may inhibit the recovery of BA from jaundice and cholangitis *via* activation of the MAPK pathway, continuous inflammatory response, inflammatory cell infiltration, as well as activation of stellate cells[40]. However, no significant difference was found in the 2-year survival rate of patients with different expression levels of RRAS. Validation may require a long-term follow-up and a larger number of subjects.

In summary, autoimmune response mediated by T lymphocytes may play a vital role in the pathogenesis of BA. The RRAS gene and its related MAPK pathway are important regulatory modules in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

ACKNOWLEDGMENTS

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COMMENTS

Background

Biliary atresia (BA) is an inflammatory obliterative cholangiopathy with unknown etiology, leading to progressive fibrosis and cirrhosis. Although there are some studies involving DNA microarrays on BA, very few studies are available on gene expression profiling of BA at different stages of its clinical course, which is why the clinicopathologic characteristics of BA vary with the age.

Research frontiers

Microarray technology and bioinformatics, emerged indispensable research tools for gene expression profiling, have been used to study the pathogenesis of BA and allow the simultaneous analysis of thousands of transcripts within a single experiment. In this study, genes that play a key role in the pathogenesis and prognosis of BA were identified.

Innovations and breakthroughs

In the current study, DNA microarrays for whole genome gene expression and bioinformatics analysis were used to characterize the differentially expressed gene patterns in normal livers and livers from BA patients at different ages, as well as to ascertain genes and pathways playing a central role in the pathogenesis of BA. The results demonstrate that *RRAS* gene and its related MAP kinase (MAPK) pathway are important regulatory modules in the pathogenesis of BA, which may serve as a novel prognostic marker for BA.

Applications

By identifying genes and pathways playing a central role in the pathogenesis of BA, this study may represent a future strategy for therapeutic intervention in treatment of BA.

Terminology

RRAS gene is a component of the MAPK signaling pathway with GTP kinase activity. The MAPK pathway is associated with BA. Consequently, it is reasonable to speculate that the RRAS gene plays an important role in the pathogenesis of BA.

Peer review

This paper is interesting and valuable for other researchers. BA is a pediatric liver disease, which can lead to liver-related death and is the most common indication for liver transplantation in children. Therefore, the early proper treatment of BA with Kasai procedure is important in this group of patients. Early diagnosis of BA and knowledge of its prognostic factors can improve the treatment outcome of BA. Different prognostic factors have been described in the literature, but no report is available on RRAS as a key regulator and an important prognostic biomarker for BA identified by DNA microarray and bioinformatics.

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BRIEF ARTICLE

HBV infection decreases risk of liver metastasis in patients with colorectal cancer: A cohort study

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Abstract

AIM: To evaluate the effect of hepatitis B virus (HBV) infection on liver metastasis of colorectal cancer.

METHODS: A total of 1298 colorectal cancer patients were recruited from January 2001 to March 2005 in this study. Enzyme-linked immunosorbent assay was used to test serum HBV markers for colorectal cancer. Patients were divided into study (infection) group and control (non-infection) group. Clinical features of patients in two groups were compared.

RESULTS: Liver metastasis was found in 319 out of the 1298 colorectal cancer patients. The incidence of liver metastasis was significantly lower in study group than in control group (14.2% *vs* 28.2%, $P < 0.01$). HBV infection significantly decreased the risk of liver metastasis [hazard ratio (HR): 0.50, 95% confidence interval (95% CI): 0.38-0.66], but the incidence of extrahepatic metastasis was significantly higher in study group than in control group (31.9% *vs* 17.0%, $P < 0.01$). The HR was the lowest in chronic hepatitis B group (HR: 0.29, 95% CI: 0.12-0.72). The number of liver metastatic lesions was significantly less in study group than in control group with a higher surgical resection rate. However, no significant difference was found in survival rate between the two groups ($P = 0.95$).

CONCLUSION: HBV infection decreases the risk of liver metastasis in patients with colorectal cancer and elevates the surgical resection rate of liver metastatic lesions.

Key words: Hepatitis B virus; Colorectal cancer; Liver metastasis; Risk

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INTRODUCTION

Colorectal cancer (CRC) accounts for 10%-15% of all cancers and is the second leading cause of cancer-related deaths in Western countries[1]. Approximately half of CRC patients develop metastatic disease[2]. Of the CRC patients, 15%-25% present with synchronous liver metastasis and 80%-90% are initially found to have unresectable liver metastatic disease[3]. Metastatic liver disease more frequently develops metachronous metastasis following treatment of CRC. It is estimated that over half of dead CRC patients have liver metastasis at autopsy[4].

Hepatitis B virus (HBV) infection is the most common cause of chronic liver diseases worldwide, an estimated 350 million persons are chronically infected with HBV worldwide, and China is a highly endemic area of HBV infection with approximately 170 million HBV carriers[5]. It has been demonstrated that HBV infection plays an important role in the development of hepatocellular carcinoma (HCC)[6]. It was reported that HBV infection finally reduces the risk of intrahepatic metastasis in HCC patients with a higher survival rate and therefore can be considered an important prognostic factor for HCC patients[7].

Rare reports are available on the relation between HBV infection and hepatic metastasis of CRC. Utsunomiya *et al*[8] reported that CRC seldom metastasizes to liver of patients infected with HBV or hepatitis C virus (HCV), but most patients in their study were infected with HCV. Song *et al*[9] showed that chronic HBV infection with viral replication reduces hepatic metastasis of CRC and prolongs the survival time of CRC patients. However, their study was hard to demonstrate the relation between HBV infection and hepatic metastasis of CRC due to its small sample size. Alternatively, investigation of experimentally induced hepatic metastasis of colon cancer demonstrated that activated immune cells residing in livers can effectively kill metastatic tumor cells, indicating that alterations in liver-associated immunity play an important role in hindering hepatic metastasis[10]. Thus, we designed this cohort study to observe the relation between HBV infection and liver metastasis of CRC.

MATERIALS AND METHODS

Patients

A total of 1298 CRC patients at the age of > 16 years, admitted to Sun Yat-Sen University Cancer Center (Guangzhou, China) from January 2001 to March 2005, were recruited in this study and divided into study (infection) group and control (non-infection) group. All patients gave their written informed consent to receive a test for HBV infection at their first visit. The study was approved by The Ethics Committee of Sun Yat-Sen University Cancer Center.

Serologic assay for viral infection

HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc were detected by enzyme-linked immunosorbent assay and HBV deoxyribonucleic acid (HBV-DNA) was detected by polymerase chain reaction.

Treatment

Primary colorectal adenocarcinoma was completely removed from all eligible patients with no prior chemotherapy or radiotherapy, and staged according to AJCC Cancer Staging Manual, 6th edition[11]. All patients received 5-fluorouracil-based *FOLFOX6* or *XELOX* regimen. Patients with liver metastasis underwent palliative treatment (including chemotherapy, radiotherapy, surgical resection and radio-frequency ablation) according to the update NCCN Guidelines for CRC[12].

Assessment of study and follow-up of patients

Patients were assessed by abdominal and pelvic computed tomography (CT) scan or magnetic resonance imaging (MRI), thoracic radiography or thoracic CT or MRI before surgery. Patients who underwent surgery were assessed again during operation. All patients, after discharged from hospital, were followed up according to a standard protocol[13]. The patients were followed up every 3 mo in the first 2 years after surgery, during which clinical examination, routine blood test, assessment of tumor markers, and abdominal ultrasonography or CT scan and endoscopy were performed. In the next 3 years, the patients were followed up every 6 mo and underwent endoscopy every 12 mo. The relapse of CRC (defined as local recurrence or metastasis at distant sites) at other sites was detected and staged. The follow-up was terminated in April 2010.

Statistical analysis

Differences in baseline clinical parameters and treatment outcomes between the two groups were evaluated by chi-square test or Student *t* test. Hazard ratio (HR) and 95% confidence interval (95% CI) were calculated with the Cox proportional-hazards model. Overall survival (OS) and disease-free survival (DFS) curves were plotted with the Kaplan-Meier method, and compared by log-rank test. OS rate was calculated from the date of discharge to death. DFS time was defined as the time between discharge and first relapse of CRC. A two-tailed *P* value less than 0.05 was considered statistically significant. Statistical analysis was performed with SPSS for Windows V.13.0.

RESULTS

The 1298 patients were divided into study group and control group. Three hundred and thirty-two patients (25.6%) with chronic HBV infection included in study group were further divided into 3 subgroups according to their natural history of HBV infection[14]. Chronic hepatitis B (CHB) was identified in 37 patients (2.9%) according to the presence of HBsAg and HBeAg or HBV-DNA which are markers of active viral replication. Inactive HBsAg carriers (IC), identified in 108 patients (8.3%), were characterized

Table 1 Baseline characteristics of patients included in this study *n* (%)

Characteristic	Study group	Control group	*P*
No. of patients	332 (100)	966 (100)	
Gender			NS
Male	196 (59.0)	550 (55.0)	
Female	136 (41.0)	416 (45.0)	
Age (yr)			NS
Median	53	60	
Range	16-81	16-87	
Depth of tumor invasion[1]	299 (100)	857 (100)	NS
T1	21 (6.3)	40 (4.1)	
T2	38 (11.4)	165 (17.1)	
T3	87 (26.2)	264 (27.3)	
T4	153 (46.1)	388 (40.2)	
Lymph-node metastasis[1]	295 (100)	843 (100)	NS
N0	160 (48.2)	490 (58.1)	
N1	87 (26.2)	216 (25.6)	
N2	48 (14.5)	137 (14.2)	
Chronic liver dysfunction[2]	39 (11.7)	65 (6.7)	< 0.05
Albumin (g/dL)	39.4 ± 5.5	39.4 ± 11.1	NS
Total bilirubin (mg/dL)	13.0 ± 5.8	12.7 ± 6.7	NS
LDH (IU/L)	191.5 ± 141.7	197.5 ± 164.1	NS
ALP (IU/L)	71.7 ± 35.5	75.6 ± 49.7	NS
GGT (IU/L)	34.7 ± 54.9	35.4 ± 55.9	NS

[1]Comparison was made only in 1156 cases after primary tumor resection, including 299 cases in tudy group and 857 cases in control group; [2]Chronic hepatitis or liver cirrhosis was clinically diagnosed according to the findings in serum chemistry, ultrasonography and computed tomography. LDH: Lactate dehydrogenase; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; NS: Not significant.

by the presence of HBsAg and anti-HBe and the absence of HBeAg or HBV-DNA. Resolved hepatitis B (RHB) observed in 187 patients (14.4%) was characterized by negative HBsAg and the presence of anti-HBc ± anti-HBs. Nine hundred and sixty-four patients (74.6%) were included in control group. No significant difference was found in sex, age, depth of tumor invasion, lymph-node metastasis, lactate dehydrogenase, γ-glutamyl transpeptidase, alkaline phosphatase, albumin, and total bilirubin between the two groups (Table 1). However, the liver function was significantly worse in study group than in control group.

Follow-up

The mean follow-up time of patients was 6 mo after operation. The median interval time of patients was 6 mo after operation. The median follow-up time of patients was 57.2 mo (range 0-110.4 mo) after operation.

Liver and extrahepatic metastasis

Liver metastasis occurred in 319 patients including synchronous liver metastasis in 193 cases and metachronous liver metastasis in 127 cases. Of the 193 patients, 39 had synchronous liver metastases. Of the 127 patients, 18 had metachronous liver metastasis. Synchronous or metachronous extrahepatic metastasis occurred in 270 patients was defined as distant metastasis but not as liver metastasis. The incidence of recurrence or metastasis to the distant sites is summarized in Table 2. The incidence of liver and extrahepatic metastasis was comparable between the two groups. The incidence of liver metastasis was significantly lower in study group than in control group (14.2% *vs* 28.2%, $P < 0.01$). The Manter-Haenzel χ^2 analysis showed that HBV infection significantly decreased the risk of liver metastasis (HR: 0.50, 95% CI: 0.38-0.66). The incidence of extrahepatic metastasis was significantly higher in study group than in control group (31.9% *vs* 17.0%, $P < 0.01$). No difference was found in liver metastasis between the two groups.

Table 2 Synchronous and metachronous metastasis in two groups *n* (%)

Sites of metastasis	Study group (*n* = 332)	Control group (*n* = 966)	*P* value	HR (95% CI)
Liver			< 0.01	0.50 (0.38-0.66)
Yes	47 (14.2)	272 (28.2)		
No	285 (85.8)	694 (71.8)		
Extrahepatic			< 0.01	1.88 (1.52-2.33)
Yes	106 (31.9)	164 (17.0)		
No	226 (68.1)	802 (83.0)		

HR: Hazard ratio; 95% CI: 95% confidence interval.

Table 3 Synchronous and metachronous metastasis in chronic hepatitis B and control groups *n* (%)

Metastatic sites	CHB group (*n* = 37)	Control group (*n* = 966)	*P* value	HR (95% CI)
Liver			< 0.01	0.29 (0.12-0.72)
Yes	3 (8.1)	272 (28.2)		
No	34 (91.9)	694 (71.8)		
Extrahepatic			< 0.01	2.55 (1.77-3.67)
Yes	16 (43.2)	164 (17.0)		
No	21 (56.8)	802 (83.0)		

CHB: Chronic hepatitis B; HR: Hazard ratio; 95% CI: 95% confidence interval.

Table 4 Synchronous and metachronous metastasis in inactive carriers and control group *n* (%)

Metastatic sites	IC group (*n* = 108)	Control group (*n* = 966)	*P* value	HR (95% CI)
Liver			< 0.01	0.36 (0.22-0.59)
Yes	11 (10.2)	272 (28.2)		
No	97 (89.8)	694 (71.8)		
Extrahepatic			< 0.01	2.24 (1.66-3.01)
Yes	41 (38.0)	164 (17.0)		
No	67 (62.0)	802 (83.0)		

IC: Inactive carriers; HR: Hazard ratio; 95% CI: 95% confidence interval.

The liver metastasis rate in patients with CHB, IC and RHB is listed Tables 3-5. CHB, IC and RHB decreased the risk of liver metastasis and increased the risk of extrahepatic metastasis. The HR was the lowest in patients with CHB (HR: 0.29, 95% CI: 0.12-0.72).

The number, size and surgical resection rate of metastatic lesions are listed in Table 6. The number of liver metastatic lesions was significantly less in study group than in control group with a higher surgical resection rate

Table 5 Synchronous and metachronous metastasis in resolved hepatitis B and control groups *n* (%)

Metastatic sites	RHB group (*n* = 187)	Control group (*n* = 966)	*P* value	HR (95% CI)
Liver			< 0.01	0.63 (0.46-0.85)
Yes	33 (17.6)	272 (28.2)		
No	154 (82.4)	694 (71.8)		
Extrahepatic			< 0.01	1.54 (1.16-2.04)
Yes	49 (26.2)	164 (17.0)		
No	138 (73.8)	802 (83.0)		

RHB: Resolved hepatitis B; HR: Hazard ratio; 95% CI: 95% confidence interval.

Table 6 Clinical features of liver metastatic lesions in two groups *n* (%)

Metastatic lesion	Study group	Control group	*P* value
Number	47	272	< 0.05
Single	17 (36.2)	73 (26.8)	
Multiple	30 (63.8)	199 (74.2)	
Size (cm)	3.6 ± 2.0	3.9 ± 1.3	NS
Resected	14 (29.8)	43 (15.8)	< 0.05

Size of liver metastatic lesions is expressed as mean ± SD. NS: Not significant.

($P < 0.05$). No significant difference was found in size of liver metastatic lesions between the two groups.

Survival rate

The 5-year survival rate was 57.0% and 58.2%, respectively, for the patients in two groups. No significant difference was found in OS and DFS rate between the two groups (Figure 1A and B).

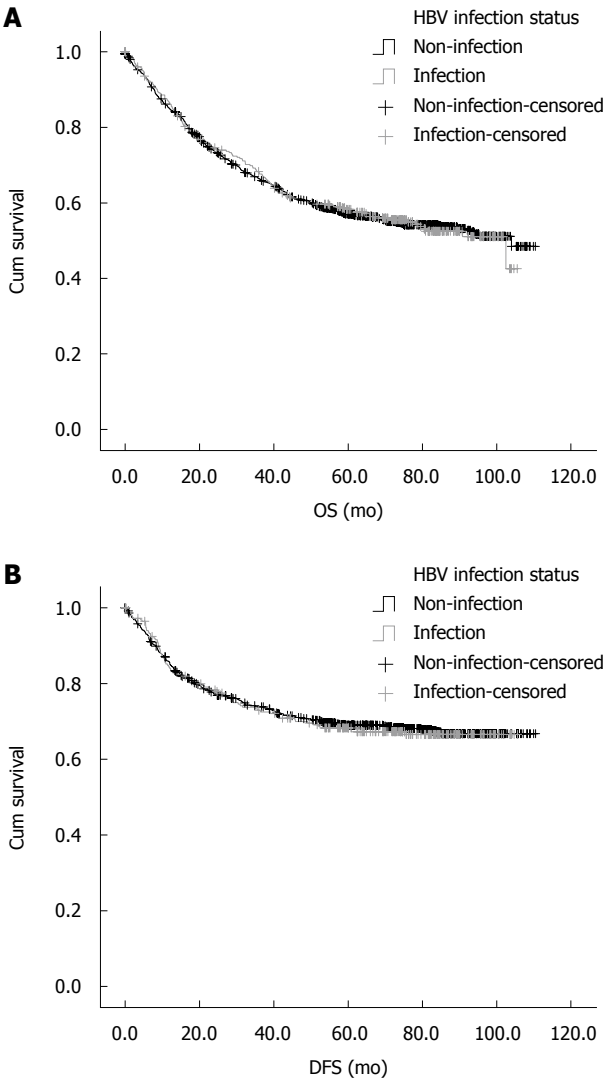

Figure 1 Overall survival rate (A) and disease-free survival rate (B) for patients in two groups after operation. HBV: Hepatitis B virus; OS: Overall survival; DFS: Disease-free survival.

DISCUSSION

In the current study, the risk of liver metastasis was significantly lower in study group than in control group (HR: 0.50, 95% CI: 0.38-0.66, $P < 0.05$). A significant difference was found in extrahepatic metastasis rate and no significant difference was found in survival rate between the two groups, suggesting that HBV infection may have a significant effect on liver metastasis of CRC. It was reported that the liver metastasis rate is low in patients with other malignancies due to HBV infection[7,15].

In this study, the liver metastasis rate of CRC was lower in CHB, IC and RHB subgroups than in control group ($P < 0.05$). CHB was characterized by positive HBeAg while the serum HBV DNA level and normal aminotransferase level were very low or undetectable in IC. RHB results from previous HBV infection without further virological, biochemical or histological evidence of active virus infection or disease[14,16]. Thus, it is reasonable to postulate that HBV infection with or without virus replication, may affect liver metastasis of CRC. In this study, CHB most significantly decreased the risk of liver metastasis of CRC followed by RHB.

Hepatic resection remains the only curative therapy for liver metastasis of CRC. In this study, the 5-year survival rate of CRC patients was 25%-40% after operation, which is consistent with the reported findings[17,18]. The number of liver metastatic lesions was much less in study group than in control group, leading to a higher surgical resection rate of liver metastatic lesions, indicating that HBV infection plays an important role in the pathogenesis of liver metastasis of CRC. However, no difference was found in overall survival and disease-free survival rate between the two groups, suggesting that liver metastasis of CRC results from the difference in extrahepatic metastasis.

Whether changes in liver-associated immunity contribute to the impediment of CRC colonization in patients infected with HBV remains unclear. The liver has a rich diversity of innate immune cells, particularly lymphocytes including natural killer cells, which respond to altered expression of self-antigens and lyse neoplastic target cells in the absence of additional activating stimuli[19]. A large number of phagocytic and antigen-presenting cells including liver sinusoidal endothelial cells, Kupffer cells and dendritic cells play an important role in local innate immunity of the liver[20]. Furthermore, it was reported

that HBV replication enhances the cytotoxicity of immunocytes during chronic HBV infection. Cytotoxic T lymphocytes (CTL) and Kupffer cells are essential for the immune response during HBV infection. HBV replication activates the specific lytic pathways of cell injury by CTL and Kupffer cells[21]. A previous study showed that the hepatic microenvironment in patients with HBV-positive metastatic liver cancer can greatly change their gene expression profiles, and the two significant clusters in the profile revealed notable changes-associated with gene products involved in immune function. In fact, over 30% of the genes in these clusters are related to this process[22]. Another study on tumor and stroma interaction suggested that the propensity of metastatic liver cancer is inherent to the tumor cells and affected by the local environment of metastatic sites[23].

In conclusion, activation of liver-associated immunity due to HBV infection reduces the incidence of liver metastasis in CRC patients.

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COMMENTS

Background

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in Western countries. Metastatic liver disease more frequently develops metachronous metastasis following treatment of CRC. It was reported that hepatitis B virus (HBV) infection finally reduces the risk of intrahepatic metastasis in hepatocellular carcinoma (HCC) patients with a higher survival rate and therefore can be considered an important prognostic factor for HCC patients Rare reports are available on the relation between HBV infection and hepatic metastasis of CRC.

Research frontiers

The authors designed a cohort study to observe the relation between HBV infection and liver metastasis of CRC.

Applications

The major points summarized in the article can applied in further studies on the correlation between liver metastasis and colorectal cancer.

Peer review

In this manuscript, the authors evaluated the effect of HBV infection on liver metastases in patients with colorectal cancer. Some discussions should be added and survival curves should be reconsidered.

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BRIEF ARTICLE

Clinical significance of K-ras and BRAF mutations in Chinese colorectal cancer patients

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Abstract

AIM: To identify and assess mutations in the *K-ras* and *BRAF* genes in a cohort of Chinese patients with colorectal cancer (CRC) for their association with various clinicopathological parameters and prognosis.

METHODS: Genomic DNA was isolated from frozen tissues. Pyrosequencing analysis was conducted to detect mutations in the *K-ras* (codons 12, 13, and 61) and *BRAF* genes (codon 600). Statistical analysis was carried out using SPSS-15.0 software.

RESULTS: Among the 118 colorectal cancer patients, we detected 41 (34.7%) mutations in the *K-ras* gene. Mutation frequencies at codon 12 and codon 13 were 23.7% (28/118) and 10.2% (12/118), respectively. Only one patient harbored a point mutation at codon 61 (0.8%, 1/118). Gender was the only factor that showed an obvious relationship with *K-ras* gene mutation (female 44.7% *vs* male 28.2%, $P = 0.037$). Other clinicopathological features, such as age, location of the tumor, tumor differentiation, Tumor, Node and Metastases classification, and the Union for International Cancer Control staging, showed no positive relationship with *K-ras* gene mutations. No significant correlation was observed between the presence of K-ras mutations (codons 12, 13, and 61) and the survival of the patients. BRAF mutations were rare, and only two patients (1.7%) harbored a detectable mutation at codon 600.

CONCLUSION: *K-ras* gene mutation is a common event in our 118 Chinese CRC patients, with an obvious relationship with gender. However, it seems not to be an independent prognostic factor in CRC patients. The *BRAF* gene is rarely mutated in Chinese CRC patients.

Key words: *K-ras*; *BRAF*; Colorectal cancer; Mutation

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world. In recent years, the morbidity and mortality of colorectal cancer has risen in the Chinese population. The development of CRC is a multistep process, which can arise due to the cumulative effect of mutations in various proto-oncogenes, tumor suppressor genes, and also from epigenetic changes in DNA. Recent evidence suggests that the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase signaling pathway, which mediates cellular responses to growth factors and regulates the elements of the cell cycle, apoptosis and differentiation[1], plays a critical role in the pathogenesis of colorectal cancer. Both the *K-ras* and *BRAF* genes encode proteins that act in the ERK signaling pathway. The K-ras proto-oncogene encodes a 21 kDa RAS protein, a member of a highly conserved family of GTPases involved in signal transduction processes. Mutations in the *K-ras* gene render the protein constitutively active in signaling by eliminating the GTPase activity. More recently, mutations in the *K-ras* gene have proved to be predictors of response to epidermal growth factor receptor-targeted therapies, such as cetuximab and panitumumab, for patients with metastatic colorectal cancer.

In human CRC, mutations in the *K-ras* gene are very frequent (20%-50%), whereas mutations of the *BRAF* gene, a downstream molecule of K-ras, occur in only 9%-11% of patients with sporadic diseases. Mutations in the *K-ras* and *BRAF* genes are frequently found to be mutually exclusive in colorectal cancer[2,3]. Both genes harbor the majority of mutations in distinct hotspots in the *BRAF* gene at codons 463-468[4] and 600[3,4], and in the *K-ras* gene at codons 12, 13[5], and, more infrequently, at codon 61[6]. Approximately 90% of the activating mutations in the *K-ras* gene are scored at codon 12 (wild-type: GGT) and codon 13 (wild-type: GGC) in exon 1, while only 5% are located at codon 61 (wild-type: CAA) in exon 2[5,7,8]. Some studies have been conducted about the relationship between *K-ras* gene mutation and various clinicopathological characteristics, but no consistent results were obtained. The prognostic significance of *K-ras* gene mutations is still controversial. As for the *BRAF* gene, very few papers regarding its prognostic significance are available in Western countries or China.

For the detection of mutations in the *K-ras* and *BRAF* genes, various techniques have been described, including temporal temperature gradient electrophoresis[9], denaturing gradient gel electrophoresis[10], restriction endonuclease-mediated selective polymerase chain reaction (PCR)[11], and direct sequencing of a PCR product[12]. For all non-sequencing methods, it is difficult to independently confirm the existence of any mutations that are identified. In addition, our previous work showed that direct sequencing of a PCR product was not a sensitive method for the detection of *K-ras* gene mutations[12]. Pyrosequencing has emerged as a sensitive and rapid sequencing method for single-nucleotide polymorphism (SNP)/mutation analysis, which overcomes the above limitations[13]. It is a non-electrophoretic, real-time sequencing technic, and a sequence-by-synthesis method that relies on the luminometric detection of pyrophosphate released upon nucleotide incorporation *via* a four-enzyme mixture reaction cascade[14,15]. It can analyze multiple samples in a short time, which makes it attractive for clinical use. So far, few papers have reported the use of pyrosequencing for the detection of *K-ras* gene mutations in colorectal cancer patients. Pyrosequencing studies on the *BRAF* gene are even fewer.

In this paper, we detected mutations in the *K-ras* and *BRAF* genes from 118 Chinese CRC patients using pyrosequencing. Correlations with various clinicopathological characteristics and the prognosis of patients were further analyzed.

MATERIALS AND METHODS

Patients and specimens

Tumor specimens used in this study were obtained from 118 CRC patients who received a radical resection operation in the 2nd Affiliated Hospital of Zhejiang University College of Medicine from February 2001 to January 2005. All patients were followed up by the Cancer Research Institute until September 2010, and the data concerning cancer recurrence and patient survival were collected. Tumor stage was classified according to the 7th edition of the Tumor, Node and Metastases (TNM) classification of the Union for International Cancer Control (UICC) staging. The clinicopathological data of all patients are shown in Table 1. Data and tissue collection was approved by the Ethics Committee of Zhejiang University College of Medicine, following the ethical guidelines of the 1975 Declaration of Helsinki.

DNA preparation and pyrosequencing

Tumor tissues were collected from the Zhejiang University Cancer Institute tissue bank. All the tissue samples were confirmed independently by two gastrointestinal pathologists. Genomic DNA was extracted with the QIAamp DNA Mini-Kit (QIAGEN, Mississauga, ON), according to the manufacturer's recommendations. The primers for the amplification and pyrosequencing assay of *K-ras* and *BRAF* gene are listed in Table 2.

PCR was performed using 100 ng genomic DNA as template. Each mixture contained 10 pmol of each primer. The reactions were performed in 1 × reaction buffer, 0.2 μmol/L dNTPs, 2 mmol/L $MgCl_2$, and 1.25 U Blend Taq polymerase (ToYoBo) in a total volume of 50 μL. The amplification reactions were as follows: an initial denaturing cycle of 94℃ for 2 min; 35 cycles of 94℃ for 30 s, 55℃ for 25 s, and 72℃ for 30 s; and a final extension cycle at 72℃ for 2 min.

The PCR products were directly subjected to the se-

Table 1 Characteristics of colorectal cancer patients in this study

Terms	*n* (%)
No. of patients	118
Median age (yr)	61
Gender	
Male	71 (60.2)
Female	47 (39.8)
Colorectal segment	
Cecum	5 (4.2)
Ascending colon	23 (19.5)
Transversal colon	8 (6.8)
Descending colon	5 (4.2)
Sigmoid	25 (21.2)
Rectum	52 (44.1)
UICC stage	
Ⅰ	18 (15.3)
Ⅱ	48 (40.7)
ⅡA	32 (27.1)
ⅡB	16 (13.6)
Ⅲ	37 (31.4)
ⅢA	5 (4.2)
ⅢB	25 (21.2)
ⅢC	7 (5.9)
Ⅳ	15 (12.7)

UICC: Union for International Cancer Control.

Table 2 Primers designed for amplification and pyrosequencing assay of *K-ras* and *BRAF* genes

	Primer sequence	Product (bp)
K-ras gene codon 12 & 13 (exon 1)	Forward: 5'-GCAGTCAACTGGAATTTTCATG-3' Reverse: 5'-biotin-GAAACCCAAGGTACATTTCAGA-3' Pyrosequencing assay: 5'-TGTGGTAGTTGGAGCT- 3'	431
K-ras gene codon 61 (exon 2)	Forward: 5'-ATCCAGACTGTGTTTCTCCCTTC-3' Reverse: 5'-biotin-ACTGCTCTAATCCCCCAAGAACT-3' Pyrosequencing assay: 5'-TATTCACGACACAGCAGGT-3'	378
BRAF gene codon 600 (exon 15)	Forward: 5'-ACAAGCCTTCAAAAATGAAGTAG-3' Reverse: 5'-biotin-ATCCAGACAACTGTTCAAACTGA-3' Pyrosequencing assay: 5'-GGTGATTTTGGTCTAACTACA-3'	362

quencing analysis using the pyrosequencing PyroMark ID system (PSQ 96 MA, Biotage AB, Sweden). For pyrosequencing, ssDNA was prepared from 40 μL biotinylated PCR product using streptavidin-coated sepharose, and 0.5 mmol/L sequencing primer was used for analysis (Table 2). Sequencing was performed with the SNP Reagent Kit (Biotage AB, Sweden) according to the manufacturer's instructions.

Statistical analysis

The Mann-Whitney *t* test, the Kruskal-Wallis test, and Fisher' test were used to evaluate the associations between the K-ras wild-type/mutation type and the clinicopathological variables of patients [Mann-Whitney *t* test for dichotomous variables (gender, metastasis *vs* no metastasis); Kruskal-Wallis test for no dichotomous variables (age, tumor region, differentiation and TNM stage). Kaplan-Meier survival analysis was performed to evaluate the relationship between K-ras wild-type/mutation type and survival of CRC patients. Calculations were carried out using the SPSS-15.0 software (SPSS Inc., Chicago, IL). *P* value less than 0.05 was regarded as statistically significant. All statistical tests were two-sided.

Table 3 Mutations of K-ras codons 12, 13, and 61 DNA detected by pyrosequencing assay

	Wild type (AA)	Point mutation (AA)	No. of mutations (%)
K-ras codon 12	GGT (Gly)	AGT (Ser)	2 (1.7)
	GGT (Gly)	GAT (Asp)	16 (13.6)
	GGT (Gly)	GCT (Ala)	2 (1.7)
	GGT (Gly)	GTT (Val)	8 (6.8)
K-ras codon 13	GGC (Gly)	GAC (Asp)	12 (10.2)
K-ras codon 61	CAA (Gln)	CAT (His)	1 (0.8)

AA: Amino acid; Gly: Glycinel; Gln: Glutamine; Ser: Serine; Asp: Aspartic acid; Ala: Alanine; Val: Valine; His: Histidine.

RESULTS

Mutation characteristics of K-ras gene

A total of 41 mutations of *K-ras* gene were detected in the 118 patients, with a mutation rate of 34.7% (41/118). And 23.7% (28/118) of mutations were at codon 12 and 10.2% (12/118) at codon 13. Only one patient harbored a point mutation at codon 61 (0.8%, 1/118). The mutations in the *K-ras* gene are summarized in Table 3. Figure 1 shows an example of pyrosequencing analysis of mutations located at codon 12, 13, and 61. Compared with the wild-type sequence of codons 12, 13, and 61 (GGT/GGC/CAA), one mutation was detected at codon 12 (Figure 1A) (GGT > GAT), one at codon 13 (Figure 1B) (GGC > GAC), and one at codon 61 (Figure 1A) (CAA > CAT).

The distribution of all the detected mutations is shown in Figure 2. The most frequently observed mutations were G-A transitions (30/41, 73.2%), followed by G-T transversions (8/41, 19.5%), two G-C transversions (2/41, 4.9%), and one A-T transversion (1/41, 2.4%). A total of 28 mutations were detected at codon 12 (wild-type GGT), representing four different mutational types. Two G-A transitions (2/28, 7.1%) were located at the first nucleotide of codon 12, resulting in an amino acid change from glycine (Gly) to serine, while the other base substitutions were all located at the second nucleotide of codon 12. The mutated GAT leading to an amino acid change from Gly to aspartic acid (Asp) (16/28, 57.1%) was the most frequently observed mutation. Eight patients were found to harbor a GTT mutation leading to an amino acid change from Gly to valine (Val) (8/28, 28.6%), while the other two patients harbored a GCT mutation (2/28, 7.1%), changing Gly to alanine. Twelve patients (12/118, 10.2%)

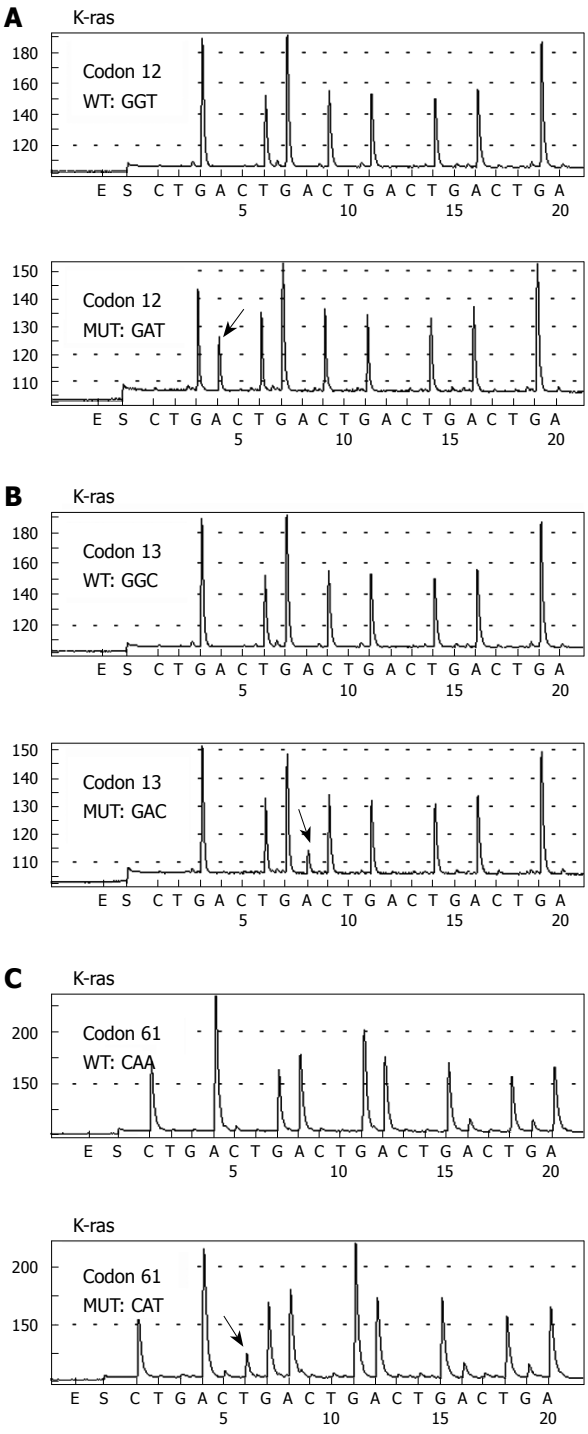

Figure 1 Pyrosequencing analysis of K-ras codons 12, 13, and 61 DNA sequences in colorectal cancer patients. The highlighted arrow shows the nucleotide change at the mutation site.

had detectable mutations at codon 13. The G-A transition was the only mutational type found, resulting in an amino acid change from Gly to Asp. Among all the 118 CRC patients, only one (1/118, 0.8%) had a detectable point mutation (A-T transversion) at codon 61, which resulted in an amino acid change from glutamine to histidine.

Correlation between K-ras gene mutations and clinicopathological features

As shown in Table 4, differences in the categorical variables, including age, gender, anatomical location of the tumor, tumor differentiation, TNM classification and UICC staging, between patients with and without K-ras mutations were evaluated for significance with χ^2 tests. Gender was the only variable that showed a significant relationship with *K-ras* gene mutation status. The prevalence of

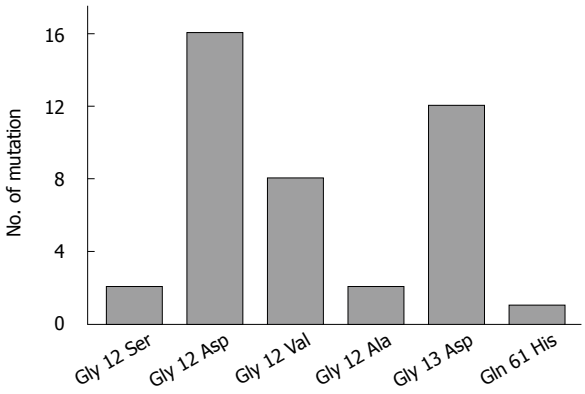

Figure 2 Distribution chart of six different K-ras mutations in 118 colorectal cancer patients. Gly: Glycinel; Gln: Glutamine; Ser: Serine; Asp: Aspartic acid; Val: Valine; Ala: Alanine; His: Histidine.

Table 4 Correlation between K-ras mutations and clinicopathological factors in colorectal cancer *n* (%)

Terms	All	Wild type	Mutation type	*P* value
No. of patients	118	77 (65.3)	41 (34.7)	
Gender				0.037
Male	71	51 (71.8)	20 (28.2)	
Female	47	26 (55.3)	21 (44.7)	
Median age (yr)	61.0	64.0	60.0	0.728
Males	65.0	65.0	65.5	
Females	60.0	60.5	58.0	
Colorectal segment				0.559
Cecum	5	1 (20.0)	4 (80.0)	
Ascending colon	23	17 (73.9)	6 (26.1)	
Transversal colon	8	4 (50.0)	4 (50.0)	
Descending colon	5	4 (80.0)	1 (20.0)	
Sigmoid	25	16 (64.0)	9 (36.0)	
Rectum	52	35 (67.3)	17 (32.7)	
Differentiation				0.761
Poor	17	12 (70.6)	5 (29.4)	
Moderate	42	25 (59.5)	17 (40.5)	
Well	59	40 (67.8)	19 (32.2)	
UICC classification				0.631
Ⅰ	18	9 (50.0)	9 (50.0)	
Ⅱ	48	37 (77.1)	11 (22.9)	
Ⅲ	37	23 (62.2)	14 (37.8)	
Ⅳ	15	8 (53.3)	7 (46.7)	
Bowel wall invasion (pT)				0.120
pT1	2	1 (50.0)	1 (50.0)	
pT2	21	11 (52.4)	10 (47.6)	
pT3	65	43 (66.2)	22 (33.8)	
pT4	30	22 (73.3)	8 (26.7)	
Lymph node metastasis (pN)				0.585
pN0	69	47 (68.1)	22 (31.9)	
pN1-2	49	31 (63.3)	18 (36.7)	
Distant metastasis (pM)				0.301
pM0	103	70 (68.0)	33 (32.0)	
pM1	15	8 (53.3)	7 (46.7)	

UICC: Union for International Cancer Control.

Table 5 Relationship in K-ras mutation between gender and tumor location

	No. of K-ras mutated patients (%)		Total No. of patients
	Colon	Rectum	
Male	12 (16.9)	8 (11.2)	71
Female	5 (10.6)	16 (34)	47

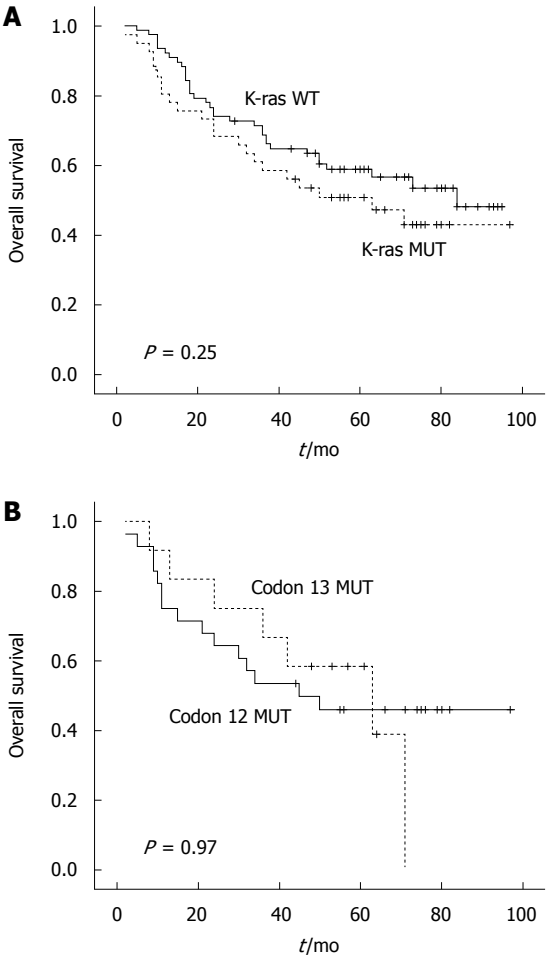

Figure 3 Kaplan-Meier survival curve in colorectal cancer patients with regard to *K-ras* gene codon mutations.

gene mutations was higher in female patients than in male patients (44.7% *vs* 28.2%, $P = 0.037$). The K-ras mutation frequency in the rectum was higher in female than in male patients (34.0% *vs* 11.2%, $P < 0.05$). Male patients had a higher mutation rate in the colon than female patients (16.9% *vs* 10.6%, $P < 0.05$) (Table 5).

Correlation of K-ras gene mutations with patient survival

As shown in Figure 3, patients with the wild-type *K-ras* gene had a median survival of 84.0 mo, which was a little longer than the patients with a mutated *K-ras* gene, whose median survival was 63.0 mo. However, this difference was not statistically significant (Figure 3A) ($P = 0.25$). The patients with a mutation at codon 12 and codon 13 had a median survival of 45.0 mo and 63.0 mo, respectively. The patient harboring a mutation at codon 61 had a rather long survival

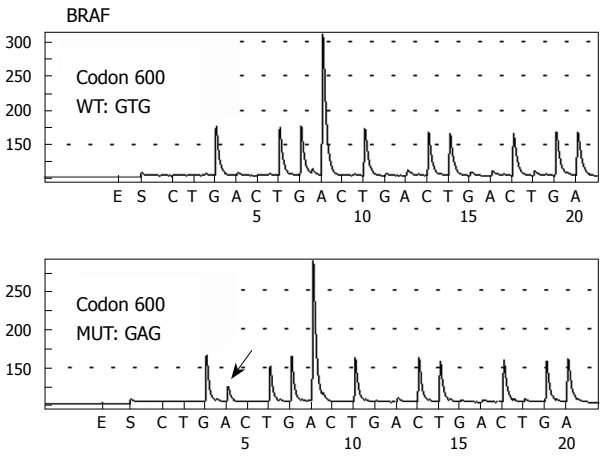

Figure 4 Pyrosequencing analysis of *BRAF* gene codon 600 DNA sequences in colorectal cancer patients. The highlighted arrow shows the nucleotide change at the mutation site.

of 73.0 mo. There were no significant correlations between the presence of K-ras mutations at codons 12, 13 and 61) and patient survival (Figure 3B).

Mutation characteristics of BRAF gene

Of the 118 CRC patients analyzed, only two patients (1.7%, 2/118) harbored a detectable mutation at codon 600 in exon 15 of the *BRAF* gene (Figure 4). Both mutations were T > A transitions (GTG > GAG) at codon 600, which resulted in an amino acid change from Val to glutamic acid (Glu). Both patients had a wild-type *K-ras* gene. One patient was a 70-year-old man with a moderately differentiated adenocarcinoma in the ascending colon at pathological stage of T3N1M0. The patient survived for 13 mo after the operation, but died of other diseases without evidence of tumor recurrence and metastasis. The other patient was a 56-year-old man. He was operated on for a well-differentiated adenocarcinoma in the transverse colon also at pathological stage of T3N1M0. Sixty-three months after operation, the patient died of tumor recurrence.

DISCUSSION

In this study, we detected various mutations of the *K-ras* and *BRAF* gene in 118 Chinese CRC patients using pyrosequencing. Mutations in the *K-ras* gene occurred very frequently (20%-50%) in CRC. It was reported in Western countries that approximately 90% of the activating K-ras mutations were found at codon 12 and codon 13 of exon 1, and about 5% at codon 61 located in exon 2. In domestic reports, with a limited number of samples, there was a similar mutation rate at codon 12 and 13, and a rather lower rate at codon 61 (0%-4.8%).

In our previous studies published in the 1990s, *K-ras* gene mutations were detected in 12 out of 35 (12/35, 34.3%) CRC patients by the PCR-RFLP method[16]. Eleven out of the 12 detected mutations (11/35, 31.4%) were located at codon 12, while the other one was at codon 61 (1/35, 2.9%). None of the patients had a mutation at codon 13. Mutations were also found in the pericancer-

ous mucosa in some cases[17], indicating that the *K-ras* gene might play an important role in the early stage of colorectal carcinogenesis. In the present study, a similar mutation rate was observed. Among the 118 patients in this study, the rates of mutated (41/118, 34.7%) and non-mutated (77/118, 65.3%) *K-ras* genes were similar to those reported by other countries. The K-ras mutation rates at codon 12, codon 13 and codon 61 were 23.7% (28/118), 10.2% (12/118) and 0.8% (1/118), respectively. Among all 41 mutations detected in *K-ras* gene in this study, 68.3% (28/41) were located at codon 12, 29.3% (12/41) at codon 13, and 2.4% (1/41) at codon 61. Similar to the previously reported data, G-A transitions were the most frequently found type of *K-ras* gene mutations in our study, followed by G-T transversions. Among the four different mutations detected at codon 12, Gly12Asp was the most frequent point mutation, accounting for about 40% of all mutations detected in the *K-ras* gene and 60% of all mutations at codon 12. All 12 point mutations detected at codon 13 were Gly13Asp. This base substitution accounted for 30% of all mutations detected in the *K-ras* gene. The distribution of the six different mutations (Figure 1) among the mutated patients was in concordance with the published data[18]. These point mutations resulting in amino acid substitution would activate RAS proteins, produce an alteration in the transduction of signals in the RAS pathway and ultimately lead to increased mitogenic signaling.

It is widely accepted that mutations in the *K-ras* gene are early events in colorectal carcinogenesis. As described by Vogelstein *et al*[5], a *K-ras* gene mutation might happen in the progression from adenoma to carcinoma. Some studies have investigated the relationship between K-ras mutation and various clinicopathological parameters, but the results remain controversial. For example, it was reported by Zlobec *et al*[19] that K-ras mutations were associated with neither clinicopathological parameters, such as gender, age, tumor location, histological type, tumor T and N stage, tumor grade and vascular invasion nor survival time of patients. Naguib *et al*[20] reported a mutation rate of 22% in the *K-ras* gene (codon 12 and 13), and a positive relationship with more advanced Dukes' stage and microsatellite stable status. In the present study, we did not find any significant correlations between the presence of K-ras mutations at codons 12, 13, and 61 or mutation type and various clinicopathological features, such as age, anatomical location of the tumor, tumor differentiation, TNM classification, and UICC staging. Gender was the only variable that showed an obvious relationship with *K-ras* gene mutation, and suggested that female patients had a higher prevalence of gene mutation than male patients (44.7% *vs* 28.2%, $P = 0.037$). Breivik *et al*[7] demonstrated that K-ras mutations were much less frequent in colon samples from male patients compared with female patients at codon 12 and 13 in Western populations. However, we found that male patients had a higher mutation rate in colon samples than female patients in the Chinese population (16.9% *vs* 10.6%, $P < 0.05$). Ethnicity, environment, and lifestyle differences may explain the difference in K-ras mutation frequency in the colon between Chinese and Western population. However, a larger population of Chinese patients is needed to confirm our findings.

Some studies have indicated the importance of K-ras alterations in predicting long-term outcome, while others have failed to show such a relationship. The collaborative RASCAL study reported a correlation between the specific K-ras mutation Gly12Val and poor prognosis, especially in Dukes' C CRC patients[21]. The Gly12Val mutation at codon 12 reported by Al-Mulla *et al*[22] and the G > A mutation at codon 13 reported by Samowitz *et al*[18] both might be related to the poor survival of the patients. On the other hand, Tortola *et al*[23] and Dix *et al*[24] failed to prove a positive relationship between K-ras mutation and shorter survival. In our study, prognostic analysis for K-ras mutations showed that none of the K-ras mutations were predictive of patient survival. Patients with a wild-type *K-ras* gene had a median survival of 84.0 mo, which was slightly longer than patients with a mutated *K-ras* gene (63.0 mo); however, this difference was not statistically significant ($P = 0.25$). There was also no significant difference in survival between patients harboring a mutation at codon 12 and those at codon 13 (45.0 mo *vs* 63.0 mo, $P = 0.97$). The shorter survival in the patients reported by Al-Mulla *et al*[22] (Gly12Val mutations at codon 12) and by Samowitz *et al*[18] (G > A mutation at codon 13), was not observed in our study. A further study with a larger number of samples will hopefully confirm the results in this study.

The K-ras/BRAF/ERK signaling pathway plays an important role in colorectal carcinogenesis. Encoding a downstream molecule of K-ras, the *BRAF* gene (codon 600) is mutated in 12%-15.6% (45/374) of colorectal carcinomas[19,20]. It was reported by Zlobec *et al*[19] that *BRAF* gene mutations are strongly associated with right-sided tumor location, higher tumor grade, absence of peritumoral lymphocytic inflammation, and microsatellite instability (MSI-H)[19]. It was also reported that a mutated *BRAF* gene is an adverse prognostic factor in right-sided colon cancer patients independent of MSI status, and in patients with lymph node-negative disease[19]. More recently, Richman[25] showed that mutations in either KRAS or BRAF are factors for poor prognosis and the overall survival (OS), and have minimal impact on progression-free survival (PFS). The mutation status of either gene does not affect the impact of irinotecan or oxaliplatin on PFS or OS. To our knowledge, this is the first paper reporting mutations in the *BRAF* gene in a large population of Chinese CRC patients. In our study, only two mutations (1.7%, 2/118) at codon 600 in exon 15 of the *BRAF* gene were detected in 118 Chinese CRC patients. Both patients had a wild-type *K-ras* gene. The mutation rate was very low compared with that reported in Western studies. Both mutations were T to A transversions (GTG > GAG), resulting in an amino acid change from Val to Glu. Both patients were male, with a Dukes' C (T3N1M0) carcinoma at the right-side of the colon. Due to the limited mutations detected, it was impossible to analyze the correlation of *BRAF* gene mutations with various clinicopathological features or prognosis. There might be a few explanations for the low incidence of *BRAF* gene mutations in our study. First, we only ana-

lyzed mutations at codon 600 in exon 15 of the *BRAF* gene. Therefore, mutations at other sites (e.g. codons 463-468) might affect the result. Second, ethnic differences might exist in CRC between Chinese and Western populations. Liao *et al*[26] found that the BRAF mutation frequency was only 4.9% in 61 Chinese colorectal tissues, which is also lower than that in Western populations. Wójcik *et al*[27] screened for mutations in exons 11 and 15 of the *BRAF* gene in 163 resected adenocarcinomas in a Polish population and only six (3.7%) tumors had a missense point mutation (G469A, D594G, G596R, K601N, and two V600E). Future studies containing a larger number of Chinese samples are expected to further clarify the result.

In conclusion, the mutation of the *K-ras* gene was a common event in our 118 Chinese CRC patients, with an obvious relationship with gender. Female patients had a higher prevalence of gene mutation than male patients. *K-ras* gene mutation seemed not to be an independent prognostic factor in CRC patients. The *BRAF* gene was rarely mutated in Chinese CRC patients.

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COMMENTS

Background

In recent years, the morbidity and mortality of colorectal cancer (CRC) has risen in the Chinese population. The *K-ras* and *BRAF* genes encode proteins that act in the extracellular signal-regulated kinase signaling pathway, which mediates cellular responses to growth factors and regulates the elements of the cell cycle, apoptosis and differentiation. Both K-ras and BRAF are prone to mutations in sporadic CRC. Mutations in K-ras could lead to constitutive activation of this pathway, resulting in cancer progression. Several recent studies have shown a strong correlation between K-ras and BRAF mutations and response to panitumumab and cetuximab.

Research frontiers

Results were in consistent among studies of the relationship between *K-ras* gene mutations and various clinicopathological characteristics. The prognostic significance of *K-ras* gene mutations is also controversial. With regard to the prognostic significance of the *BRAF* gene, less information is available in both Western and Chinese populations.

Innovations and breakthroughs

Recent reports have highlighted the importance of *K-ras* and *BRAF* gene mutations, the clinicopathological characteristics of CRC and its response to epidermal growth factor receptor-targeted therapies. Pyrosequencing is a powerful, sensitive and rapid sequencing method for single-nucleotide polymorphism (SNP)/mutation analysis. This is the first study to report mutations of *K-ras* and *BRAF* genes in a large population of Chinese CRC patients using pyrosequencing.

Applications

Using pyrosequencing technology, the authors found that *K-ras* gene mutations were common in Chinese CRC patients, with an obvious relationship with gender. The *BRAF* gene was rarely mutated in Chinese CRC patients.

Terminology

Pyrosequencing: Pyrosequencing is a method of determining the order of nucleotides in DNA based on the "sequencing by synthesis" principle. It relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. It is a powerful, sensitive and rapid sequencing method and can be used for DNA SNP/mutation analysis.

Peer review

This paper presents new results on the frequency of K-ras and BRAF mutations in colorectal carcinomas of Chinese patients.

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Meetings

Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 04-05, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 01, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 03-05, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 07-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 06-07, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 07-09, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 07-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-02, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 02, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 01-04, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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15 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/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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