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World Journal of Gastroenterology

Contents

Weekly Volume 20 Number 5 February 7, 2014

TOPIC HIGHLIGHT

1127 Novel methylxanthine derivative-mediated anti-inflammatory effects in inflammatory bowel disease
Lee IA, Kamba A, Low D, Mizoguchi E

1139 When combination therapy isn't working: Emerging therapies for the management of inflammatory bowel disease
Krishnareddy S, Swaminath A

1147 Prevention of post-operative recurrence of Crohn's disease
Vaughn BP, Moss AC

1155 Role of capsule endoscopy in inflammatory bowel disease
Kopylov U, Seidman EG

1165 Intestinal barrier in inflammatory bowel disease
Antoni L, Nuding S, Wehkamp J, Stange EF

1180 Magnetic resonance imaging in children and adolescents with chronic inflammatory bowel disease
Mentzel HJ, Reinsch S, Kurzai M, Stenzel M

1192 Role of the gut microbiota in inflammatory bowel disease pathogenesis: What have we learnt in the past 10 years?
Hold GL, Smith M, Grange C, Watt ER, El-Omar EM, Mukhopadhya I

1211 Therapy with stem cells in inflammatory bowel disease
Martínez-Montiel MP, Gómez-Gómez GJ, Flores AI

1228 Inflammatory bowel disease: An increased risk factor for neurologic complications
Morís G

1238 Epidemiological studies of migration and environmental risk factors in the inflammatory bowel diseases
Ko Y, Butcher R, Leong RW

1248 What is left when anti-tumour necrosis factor therapy in inflammatory bowel diseases fails?
Lawrance IC

1259 *Campylobacter concisus* and inflammatory bowel disease
Zhang L, Lee H, Grimm MC, Riordan SM, Day AS, Lemberg DA

REVIEW

1268 Novel hepatocellular carcinoma molecules with prognostic and therapeutic potentials
Scaggiante B, Kazemi M, Pozzato G, Dapas B, Farra R, Grassi M, Zanconati F, Grassi G

ORIGINAL ARTICLE

1289 Factors correlating with acoustic radiation force impulse elastography in chronic hepatitis C
Nishikawa T, Hashimoto S, Kawabe N, Harata M, Nitta Y, Murao M, Nakano T, Mizuno Y, Shimazaki H, Kan T, Nakaoka K, Takagawa Y, Ohki M, Ichino N, Osakabe K, Yoshioka K

BRIEF ARTICLE

1298 Antinociceptive effects of novel melatonin receptor agonists in mouse models of abdominal pain
Chen C, Fichna J, Laudon M, Storr M

1305 Bax protein may influence on the invasion of colorectal cancer
Pryczynicz A, Gryko M, Niewiarowska K, Cepowicz D, Ustymowicz M, Kemona A, Guzińska-Ustymowicz K

1311 Reduced hemoglobin and increased C-reactive protein are associated with upper gastrointestinal bleeding
Tomizawa M, Shinozaki F, Hasegawa R, Togawa A, Shirai Y, Ichiki N, Motoyoshi Y, Sugiyama T, Yamamoto S, Sueishi M

1318 Risk factors for proximal migration of biliary tube stents
Kawaguchi Y, Ogawa M, Kawashima Y, Mizukami H, Maruno A, Ito H, Mine T

1325 A new hemostatic clip for endoscopic surgery that can maintain blood flow after clipping
Nam KW, Lee SB, Kim IY, Kim KG, Park SJ

1332 Evaluation of specific fecal protein biochips for the diagnosis of colorectal cancer
Wang HP, Wang YY, Pan J, Cen R, Cai YK

1340 Endoscopic ultrasonography for staging of T1a and T1b esophageal squamous cell carcinoma
He LJ, Shan HB, Luo GY, Li Y, Zhang R, Gao XY, Wang GB, Lin SY, Xu GL, Li JJ

1348 Oleanolic acid and ursolic acid inhibit proliferation in transformed rat hepatic oval cells
Han YY, Xue XW, Shi ZM, Wang PY, Wu XR, Wang XJ

CASE REPORT

1357 A trichobezoar in a child with undiagnosed celiac disease: A case report
Irastorza I, Tutau C, Vitoria JC

1361 Nodular fasciitis in the mesentery, a differential diagnosis of peritoneal carcinomatosis
Shiga M, Okamoto K, Matsumoto M, Maeda H, Dabanaka K, Namikawa T, Uemura S, Munekage M, Kobayashi M, Hanazaki K

1365 Epstein-Barr virus-associated lymphoepithelioma-like early gastric carcinomas and endoscopic submucosal dissection: Case series
Lee JY, Kim KM, Min BH, Lee JH, Rhee PL, Kim JJ

1371 Bowel mesentery (meso-appendix) microcystic/reticular schwannoma: Case report and literature review
Tang SX, Sun YH, Zhou XR, Wang J

LETTERS TO THE EDITOR

1377 Pericyst: The outermost layer of hydatid cyst
Golzari SEJ, Sokouti M

World Journal of Gastroenterology
Volume 20 Number 5 February 7, 2014

Contents

APPENDIX	I-VI	Instructions to authors
ABOUT COVER		Editor-in-Chief of *World Journal of Gastroenterology*, Saleh A Naser, PhD, Professor, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32816, United States
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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Novel methylxanthine derivative-mediated anti-inflammatory effects in inflammatory bowel disease

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Abstract

Family 18 chitinases have a binding capacity with chitin, a polymer of N-acetylglucosamine. Recent studies strongly suggested that chitinase 3-like 1 (CHI3L1, also known as YKL-40) and acidic mammalian chitinase, the two major members of family 18 chitinases, play a pivotal role in the pathogenesis of inflammatory bowel disease (IBD), bronchial asthma and several other inflammatory disorders. Based on the data from high-throughput screening, it has been found that three methylxanthine derivatives, caffeine, theophylline, and pentoxifylline, have competitive inhibitory effects against a fungal family 18 chitinase by specifically interacting with conserved tryptophans in the active site of this protein. Methylxanthine derivatives are also known as adenosine receptor antagonists, phosphodiesterase inhibitors and histone deacetylase inducers. Anti-inflammatory effects of methylxanthine derivatives have been well-documented in the literature. For example, a beneficial link between coffee or caffeine consumption and type 2 diabetes as well as liver cirrhosis has been reported. Furthermore, theophylline has a long history of being used as a bronchodilator in asthma therapy, and pentoxifylline has an immuno-modulating effect for peripheral vascular disease. However, it is still largely unknown whether these methylxanthine derivative-mediated anti-inflammatory effects are associated with the inhibition of CHI3L1-induced cytoplasmic signaling cascades in epithelial cells. In this review article we will examine the above possibility and summarize the biological significance of methylxanthine derivatives in intestinal epithelial cells. We hope that this study will provide a rationale for the development of methylxanthine derivatives, in particular caffeine, -based anti-inflammatory therapeutics in the field of IBD and IBD-associated carcinogenesis.

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Key words: Adherent-invasive *Escherichia coli*; Chitinase 3-like 1; Chitinase inhibitors; Intestinal epithelial cells; Host-microbial interactions; Inflammatory bowel disease

Core tip: The involvement of family 18 chitinases in the pathogenesis of inflammatory bowel disease has been increasing characterized. The discovery of methylxanthine derivatives as an effective inhibitor of family 18 chitinases provides a good tool to control the pathogenic effects of these proteins. This review discusses the underlying inhibitory mechanisms of the different methylxanthine derivatives and how these compounds have been shown to be effective in the amelioration of animal colitis models. As such, this mode of application can be extended to target other family 18 chitinases associated disorders such as asthma.

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INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a group of intestinal inflammatory disorders that affect millions people worldwide. IBD is associated with increased risk of colorectal cancer 8-10 years after initial diagnosis[1]. The chronic colitis in IBD is asssociated with inappropriate activation of the immune system by abnormal interactions between host and enteric luminal microbes. Our group have previously identified an unexpected role for chitinase 3-like 1 (CHI3L1) in enhancing bacterial adhesion and invasion on/into intestinal epithelial cells (IECs) and have demonstrated that CHI3L1 specifically activates protein kinase B (AKT) phosphorylation in IECs[2,3]. Given these roles, the ability of a host to produce CHI3L1 and other enzymatic active mammalian chitinases [*e.g.*, chitinase-1 and acidic mammalian chitinase (AMCase)] could be a critical factor in regulating the innate immune responses against microorganisms that exist in normal intestinal flora[4]. However, exaggerated production of these chitinases could cause highly pathogenic effects in mucosal tissues, directly initiating and perpetuating chronic inflammation[2,5-7]. CHI3L1 also has been identified as a potential autoantigen driving T cell-mediated immune responses in rheumatoid arthritis, suggesting that mammalian chitinases are highly associated with chronic inflammation[8,9].

As shown by Rao *et al*[10], methylxanthine derivatives, including caffeine, theophylline and pentoxifylline, are competitive inhibitors against a family 18 chitinase expressed by a fungal pathogen. Crystallographic analysis of chitinase and methylxantine derivative complexes revealed specific interactions with the active site of the chitinase protein, mimicking the binding of allosamidin, a well-known pan-chitinase inhibitor isolated from *Streptomyces* species[10]. Currently, most known family 18 chitinase inhibitors are natural products, including pseudotrisaccharide allosamidin[11]. However, this inhibitor is unsuitable as a therapeutic lead because of its high cost and high molecular weight. In contrast, methylxanthine derivatives are inexpensive and have much lower molecular weight as compared to allosamidin. In particular, caffeine is found in a wide variety of foods and beverages (*e.g.*, coffee, tea, cola, chocolates) and dietary supplements/ingredients (including botanicals such as guarana, yerba mate, and green tea extract)[11]. At physiological concentration, caffeine shows only minor adverse effects on the cardio-respiratory system and other health outcomes[12-14]. Therefore, caffeine is thought to be the most reasonable, least expensive, and safest compound among known chitinase inhibitors. In fact, our group recently demonstrated the beneficial effects of a medium dose of caffeine (2.5 mmol/L; equivalent to the concentration of caffeine in 2-3 cups of coffee) in the development of acute dextran sulfate sodium (DSS)-induced colitis by down-regulating the expression of CHI3L1 in the colon[15]. Although anti-inflammatory effects of caffeine is considered to be mediated, at least partially, *via* chitinase inhibition, it is still largely unknown whether the other methylxanthine derivatives, such as theophylline and pentoxifylline, also exert their anti-inflammatory activities by downregulating CHI3L1 expression. In this review article, we will discuss the important biological functions of caffeine, theophylline and pentoxifylline laying a special emphasis on the CHI3L1-mediated AKT/β-catenin signaling activation in IECs.

CHI3L1, BACTERIAL INFECTION AND IBD

It has been postulated that dysregulated host-microbial interactions play a central role in the development of intestinal inflammation[16-18]. In humans, the ileocecal region and colon are colonized by a group of anaerobic bacteria, many of which cannot be cultured using standard microbial techniques[19]. Altered epithelial barrier functions, mucosal immune responses and microbial defense are major factors of host susceptibility against these commensal bacteria[19]. Therefore, abnormal adhesion and invasion of commensal bacteria on/into IECs may be highly involved in the pathogenesis of IBD in patients with the mutations in IBD-susceptibility genes[20,21]. The development of excess bacterial adhesion and/or perpetuation of intestinal inflammation seems to be closely associated with the induction of several molecules on IECs[22,23].

Previous studies have addressed the possibility that chronic bacterial infections are involved in the pathogenesis of IBD[24-26]. An involvement of *Escherichia coli* (*E. coli*) in the pathogenesis of CD has been suggested by the detection of *E. coli* antigens and DNA in granulomatous and peri-ulcerative lesions in CD[27]. In addition, circulating antibodies against the porin protein C of *E. coli* outer membrane have been detected in CD patients with severe inflammation[28]. In fact, the terminal ileum of CD patients is sometimes heavily colonized by a special type of *E. coli* strain, adherent-invasive *E. coli* (AIEC), which is able to survive extensively within IECs and macrophages without inducing apoptosis[29-32]. Interestingly, AIEC can be detected only in 6% of ilea in healthy individuals, but is present in 36% of the newly formed terminal ilea (with early and acute inflammation) of post-surgical patients[31]. It has been demonstrated by Carvalho *et al*[33] that abnormal expression of specific host receptor, carcinoembryonic antigen-related cell adhesion molecule 6, is one of the inducible molecules enhancing the interaction between host cells and AIEC[32,33].

Utilizing DNA microarray analysis, our group also identified that CHI3L1 is specifically up-regulated on IECs under intestinal inflammatory conditions. Although

Table 1 Methylxanthines compared with common chitinase inhibitors in IC_{50}

Compound name	Chemical formula	IC_{50}	Ref.
Caffeine	$C_8H_{10}N_4O_2$	469 ± 23 μmol/L against *Aspergillus fumigatus* (*A. fumigatus*) chitinase	[10]
Pentoxifylline	$C_{13}H_{18}N_4O_3$	126 ± 7 μmol/L against *A. fumigatus* chitinase	[10]
Theophylline	$C_7H_8N_4O_2$	1500 ± 90 μmol/L against *A. fumigatus* chitinase	[10]
Allosamidin	$C_{25}H_{42}N_4O_{14}$	10 μmol/L against *Candida albicans* chitinase	[42]
Argifin	$C_{29}H_{41}N_9O_{10}$	3.7 μmol/L against *Lucilia cuprina* (*L. cuprina*) chitinase	[78]
Argadin	$C_{29}H_{42}N_{10}O_9$	3.4 nmol/L at 20 ℃ against *L. cuprina* chitinase	[79]

IC_{50}: The half maximal inhibitory concentration.

CHI3L1 entirely lacks glycohydrolase enzymatic activity, it has a functional chitin-binding motif acting as chilectin[34,35]. Chitin is an *N*-acetylglucosamine polymer and is the second most abundant polysaccharides in nature next to cellulose. In spite of lacking of chitin and chitin synthase, mammals can constitutively or inducibly produce several chitinases, including CHI3L1, which show a high degree of sequential homology to the bacterial and plant chitinases[36]. The expression of CHI3L1 is highly up-regulated in IECs and macrophages with inflammation and specifically enhances potentially pathogenic, but not non-pathogenic, bacterial adhesion and invasion on/into IECs[2,37]. Our recent studies further revealed that a specific adhesion between CHI3L1 and 5 distinct amino acids in the AIEC Chitinase A (ChiA) protein, which includes chitin-binding domains (CBDs), play critical roles in the initial host-microbial interaction[7]. Furthermore, *N*-glycosylation of a single amino acid residue (68th Asparagine) in the mouse CHI3L1 protein is crucial for the adhesion of potentially pathogenic *E. coli* on IECs[7]. Interestingly, similar to CHI3L1, bacterial CBDs have been found to bind directly to chitin[38,39]. Therefore, the specific interaction between glycosylated CHI3L1 and *E. coli* ChiA seems to be enhancing the bacterial adhesion and invasion on/into IECs under inflammatory conditions. These excess and abnormal host-microbial interactions *via* the above two chitinases may further perpetuate chronic intestinal inflammation as well as colitis-associated carcinogenic change of IECs, presumably by interacting with toll-like receptor-4 signaling[40,41].

METHYLXANTHINE DERIVATIVES AS PAN-CHITINASE INHIBITORS

Methylxanthines are a group of alkaloid chemicals which are derived from the purine base xanthine. Xanthine is a result of purine degradation from either guanine by guanine deaminase or hypoxanthine by xanthine oxidoreductase. Methylxanthines are methylated derivatives and include the compounds caffeine, aminophylline, 3-isobutyl-1-methylxanthine, paraxanthine, pentoxifylline, theobromine (found in chocolate), theophylline. Traditionally, they are used as stimulants, to increase athletic performance, and as brochiodilators, most notably in the case of asthma.

Through the use of drug screening tools, it was demonstrated that several methylxanthine derivatives, namely caffeine, theophylline, and pentoxifylline were potential chitinase inhibitors[10]. Subsequent analysis confirmed the inhibitory effects of all 3 methylxanthines, with pentoxifylline having the highest K_i of 37 μmol/L. In terms of another parameter IC_{50} (half maximal inhibitory concentration), pentoxifylline was almost 4 fold lower, and thus 4 times more effective, than caffeine (126 μmol/L *vs* 469 μmol/L, respectively), and was almost 12 times more powerful than theophylline (1500 μmol/L)[10]. In contrast, allosamidin is reported of having an IC_{50} of 10 μmol/L towards *Candida albicans*-derived chitinase[42]. Therefore, methylxanthines including pentoxifylline, caffeine and theophylline do show a significantly lower affinity against fungal chitinase as compared to allosamidin. The inhibitory strength of methylxanthines compared with common chitinase inhibitors is summarized in Table 1.

Interestingly, the chemical structures involved in binding between methylxanthines and the family 18 chitinases were found to be very similar for all 3 compounds (caffeine, pentoxifylline and theophylline) and mimicked chitinase binding to allosamidin. X-ray diffraction analysis revealed a common position for the methylxanthine substructure. The additional inhibition by pentoxifylline is suspected to be due to increased interactions including hydrogen bonding, and extensive π-π stacking with the active site[10].

Methylxanthines have the potential to be very useful as chitinase inhibitors and disease treatments as summarized in Table 2. They are typically very safe in low doses and represent a class of favorable drugs due to low cost, low molecular weight, and easy availability. As demonstrated, they exhibit significant biological activity against mammalian chitinases which have been implicated in several inflammatory disorders and cancers. Therefore, their use as immune-modulators will surely provide new therapeutic approaches.

ANTI-INFLAMMATORY EFFECTS OF CAFFEINE, THEOPHYLLINE, AND PENTOXIFYLLINE

The major anti-inflammatory effects of caffeine, pentoxifylline, and theophylline result from 2 main mechanisms; the non-selective inhibition of phosphodiesterases (PDEs) and as a non-selective adenosine receptor antagonist. Through the inhibition of PDEs, a rise in intracellular cyclic adenosine mono-phosphate, activation of protein kinase A, inhibition of tumor necrosis factor alpha (TNFα) and leukotriene synthesis, and reductions in in-

Table 2 Major biological effects of methylxanthine derivatives

Compound	Molecular weight (g/mol)	Biological effects	Side effects
Caffeine	194.19	Increases alertness, slightly increases metabolic rate[80], increases blood pressure, is a diuretic, improves sports performance[81]	Caffeine dependency, restlessness, insomnia, and anxiety at high levels (250-500 mg daily[82]) In extreme amounts (> 600 mg daily chronically): Gastrointestinal (GI) disturbance[1], irregular/rapid heartbeat, mania, depression, and psychosis[83]
Pentoxifylline	278.31	Improves blood circulation through peripheral blood vessels, prevents nausea/ altitude sickness, improves red blood cell deformability (*i.e.*, sickle cell anemia), reduces blood viscosity, reduces formations of platelet aggregation/thrombus[84]	Irregular heartbeat, chest pain, dizziness, edema in extremities Acute toxicity in rats determined at 1772 mg/kg[85]
Theophylline	180.164	Relaxes bronchial smooth muscle, increases heart contractility, rate, and efficiency, increases blood pressure, increases renal circulation, stimulates respiratory center of CNS, treatment for COPD, asthma, infant apnea[86,87]	Interactions with many drug (cimetidine and phenytoin), and causes nausea, arrhythmias, insomnia, irritability, dizziness, seizures and tachyarrhythmias at toxic concentrations (> 20 mg/mL)[86]

[1]GI disturbance is marked by nausea, vomiting, abdominal pain, diarrhea, bowel incontinence, and anorexia. COPD: Chronic obstructive pulmonary disease; CNS: Central nervous system.

flammation and innate immunity are observed[43-46]. Deree *et al*[43] reported that pentoxifylline successfully reduced TNFα production after human mononuclear cells were stimulated with lipopolysaccharide. A similar result was found in peripheral blood monocytes and alveolar macrophages from sarcoidosis patients, in which pentoxifylline also inhibited the spontaneous TNFα production associated with this disease[44]. Therefore these compounds may be useful in reducing LPS-induced inflammation and as a treatment for sarcoidosis. Methylxanthine derivatives demonstrate non-selective inhibition of all PDEs by competitive inhibition and therefore they likely bind to the active site of PDEs, however their exact molecular mechanism of inhibition is still uncertain. Caffeine and theophylline were shown to inhibit several PDE isozymes to a similar extent, and the two compounds showed an almost equal affinity for each of the PDE isozymes[47].

Through the inhibition of leukotrienes, which are known as pro-inflammatory mediators involved in asthma and bronchoconstriction and which play pivotal roles in innate immunity, asthma symptoms are relieved and inflammation is reduced[46]. Leukotrienes enhance inflammation by increasing leukocyte infiltration, phagocyte microbial ingestion, and generation of pro-inflammatory cytokines including IL-5, TNFα, and macrophage inflammatory protein-1β[48]. It was proven that theophylline effectively reduced leukotrience synthesis and reduced chemotaxis of complement 5a-and platelet-activating factor-stimulated human eosinophils obtained from normal and atopic donors[49].

As a non-selective receptor antagonist for adenosine, methylxanthine derivatives, most notably, caffeine, are well known as wakefulness aids, as adenosine is a known inducer of sleep. Caffeine, theophylline, and pentoxifylline non-selectively affect several adenosine receptors, including A_1, A_{2A}, A_{2B}, and A_3. A_1 receptor is found ubiquitiously throughout the body and studies demonstrated inhibition of this receptor with the novel compound *L*-97-1 [3-[2-(4-aminophenyl)-ethyl]-8-benzyl-7-{2-ethyl-(2-hydroxy-ethyl)-amino]-ethyl}-1-propyl-3,7-dihydro-purine-2,6-dione] reduce histamine and/or adenosine-induced hyperresponsiveness and early and late allergic responses in a rabbit model of house dust mite-induced allergic reactions[50]. The A_{2A} receptor is similar to the A_1 receptor in that it is found throughout the body. Mice deficient in A_{2A} receptor had significantly higher levels of the pro-inflammatory cytokines TNFα, IL-12 p40, and IL-6 in an LPS-induced model of septic shock as compared to wild-type mice. Therefore, it is suggested that this receptor plays a pivotal role in controlling excess inflammation/tissue damage[51]. In a mouse model of allergic asthma induced by AMP or 5-*N*-ethylcarboxamidoadenosine, antagonizing the A_{2B} receptor with the compound CVT-6883 resulted in decreased cellular infiltration in bronchoalveolar lavage fluid including eosinophils and lymphocytes and reduced bronchoconstriction. Interestingly, a similar but slightly blunted response was also seen in theophylline treatment at 36 mg/mL aerosolization for 5 min[52]. Therefore, this receptor is a target for asthma patients and CVT-6883 is currently undergoing clinical trials.

A novel area of investigation of methylxanthine derivatives is the anti-inflammatory effects through the inhibition of mammalian chitinases, including CHI3L1. As previously discussed, methylxanthine derivatives are effective pan-chitinase inhibitors[10]. CHI3L1 has been shown to play a role in many inflammatory disorders including rheumatoid arthritis, asthma, hepatitis, and IBD[2,6]. CHI3L1 increases inflammation in human bronchial epithelium by inducing IL-8 and activating the MAPK and nuclear factor-κB pathways, which are involved in cell survival[53]. IL-8 inhibition was hypothesized to be an effective treatment for asthma-related inflammation/remodeling. In a model of DSS-induced colitis, caffeine treatment at 2.5 mmol/L was shown to decrease TNFα, INFγ, IL-4 in mesenteric lymph nodes, and IL-17F in mesenteric lymph nodes and colon and increased the anti-inflammatory IL-10 production in spleen, mes-

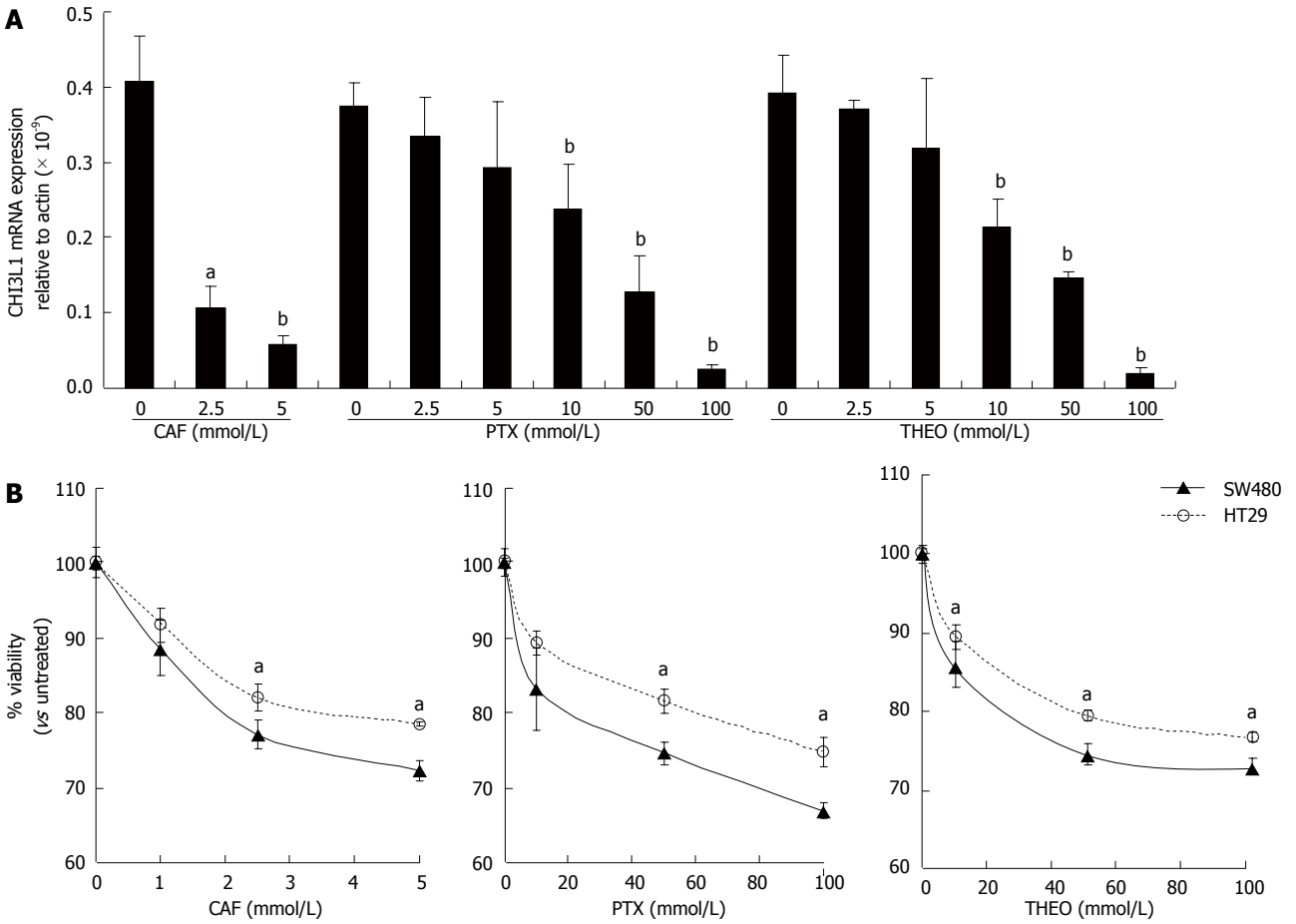

Figure 1 Caffeine, pentoxifylline and theophylline down-regulate chitinase 3-like 1 mRNA expression and reduce cell viability in human colonic epithelial cells. A: SW480 cells were stimulated with caffeine (CAF) at 0, 2.5 or 5 mmol/L or pentoxifylline (PTX) or theophylline (THEO) at 0, 2.5, 5, 10, 50 or 100 mmol/L for 48 h and detected for the chitinase 3-like 1 (CHI3L1) mRNA expression by quantitative-polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control; B: SW480 and HT29 cells were treated with either CAF (0, 1, 2.5 or 5 mmol/L), PTX or THEO (0, 10, 50 or 100 mmol/L) for 48 h and cell viability were determined using trypan blue exclusion test. CAF, PTX and THEO were purchased from Sigma-Aldrich (St Louis, MO, United States). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group.

enteric lymph nodes, and colon[7,15].

Taken together, methylxanthine derivatives have demonstrated efficacy against the inflammatory disorders, and were shown to reduce inflammation in mice treated with DSS. Therefore, the efficacy of methylxanthine derivatives as potential anti-inflammatory and anti-cancer agents should be further elucidated in other inflammatory conditions and inflammation-associated cancers.

EFFECTS OF METHYLXANTHINE DERIVATIVES ON IBD MOLECULAR PATHWAYS ASSOCIATED WITH CHI3L1

The pathological involvement of CHI3L1 in many diseases, including autoimmune diseases (*e.g.*, IBD, asthma and RA), as well as many forms of solid tumors (*e.g.*, colorectal cancer) are becoming increasingly apparent at this time. The most direct evidence is a significant amount of CHI3L1 induction during the disease state (*e.g.*, IBD and IBD-associated cancer) which activates several important cellular pathways, including AKT and the β-catenin signaling pathway, thus playing crucial roles in disease pathogenesis[2,3,6,54]. Characterization of these CHI3L1-mediated pathological pathways can facilitate a better understanding on the molecular mechanisms behind how methylxanthine derivatives can ameliorate diseases through the inhibition of CHI3L1.

In addition to direct protein inhibition of the family 18 chitinases, as determined by X-ray crystallography, *in vitro* methylxanthine treatment in SW480 colonic epithelial cells (CECs), a human colon cancer cell line, directly results in a down-regulation of CHI3L1 mRNA levels (Figure 1A)[15]. The effective dose of caffeine that is optimal for achieving such down-regulation ranges from 2.5 to 5 mmol/L (Figure 1A). Nevertheless, it was previously shown that 1.0 mmol/L caffeine treatment is sufficient to cause a down-regulation of CHI3L1 in SW480 CECs[15]. Caffeine treatment also results in the down-regulation of other mammalian chitinases including AMCase, but not chitinase 1[15]. The effective dose of pentoxifylline and theophylline to down-regulate CHI3L1 in SW480 cells ranges from 10 to 100 mmol/L, whereby any concentration below that did not show any effects on CHI3L1

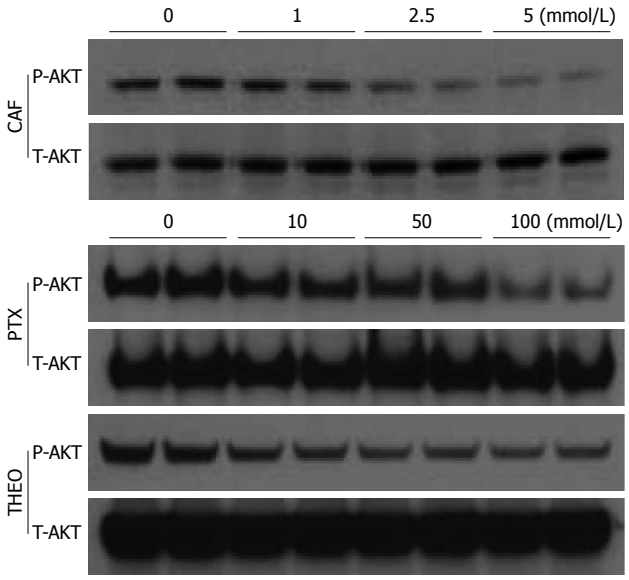

Figure 2 Caffeine, pentoxifylline and theophylline suppress protein kinase B signaling pathway activation in mouse colonic epithelial cells. CMT93 mouse colonic epithelial cells were stimulated with caffeine (CAF) (0, 1, 2.5 or 5 mmol/L), pentoxifylline (PTX) or theophylline (THEO) (0, 10, 50 or 100 mmol/L) for 48 h. Twenty five micro grams of total protein were resolved using SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using anti-phospho/total protein kinase B (AKT) Abs purchased from cell signaling technology (Danvers, MA, United States) .

mRNA expression (Figure 1A). Since rabbit anti-CHI3L1 antibody administration to mice has been shown to have an ameliorating effect in acute DSS-induced colitis development, the direct down-regulation of CHI3L1 using methylxanthine derivatives also achieves a similar therapeutic effect in IBD *in vivo*[2,15].

Furthermore, methylxanthine derivative treatment also reduces colon cancer cell viability in a CHI3L1 expression dependent manner. *In vitro* treatment with caffeine (1-5 mmol/L), pentoxifylline (10-100 mmol/L) or theophylline (10-100 mmol/L) in SW480 cells that express high endogenous CHI3L1 greatly reduces the viability of cells (Figure 1B). However, methylxanthine derivative treatment in HT29 CECs, a human colon cancer cell line that does not express endogenous CHI3L1, has minimal effect on cell viability, indicating a direct involvement of cell survival that is mediated by CHI3L1 expression, at least in part. This has important implications in carcinogenesis since many solid tumors, including colorectal cancer and breast cancer, exhibit exaggerated expression of CHI3L1[6]. Mechanistically, CHI3L1 directly contributes to tumorigenesis by exerting excessive cell proliferation and angiogenesis[54,55]. Thus, methylxanthine derivative treatment provides a proof-of-concept in controlling carcinogenic changes and progression by regulating cell viability *via* targeting ectopic CHI3L1 expression and function.

Several studies have demonstrated that AKT signaling is up-regulated in the IEC crypts of chronic UC and CD patients, as well as in a murine DSS-induced colitis model[56,57]. In contrast, colitis patients that had undergone 5-aminosalicylic acid (5-ASA) treatment showed reduced AKT-phosphorylation in inflamed tissues, suggesting a direct relationship between AKT signal activation and disease severity[58]. A progressive increase in the densities of phosphorylated AKT in tumor-associated macrophages was observed in normal, colitic and dysplastic to cancer patient specimens[59]. This expression pattern is in parallel to that of colonic CHI3L1 levels, which showed almost undetectable expression in normal colon, but is induced during colitis that further up-regulates during colitis-associated cancer development[2,54]. CHI3L1 can directly activate colonic AKT signaling, specifically *via* the 325th-339th amino acid residues within the chitin-binding motif[3]. This enhanced up-regulation of CHI3L1 during colitis-associated cancer development may provide a plausible explanation for the exaggerated enhancement of AKT phosphorylation. In the context of tumorigenesis, activation of this AKT signaling in the colon induces proliferative signals in IECs that is critical for G1 cell cycle progression[60]. Thus such constitutive activation of AKT, at least in part mediated by CHI3L1, might result in the uncontrolled cell proliferation. With this in mind, reducing AKT activation by targeting CHI3L1 using methylxanthines seemed to be a possible therapeutic strategy for inflammatory disorders. The combinatory effect of CHI3L1 protein inhibition, as well as direct down-regulation of CHI3L1 mRNA expression by caffeine, pentoxifylline and theophylline, was shown to significantly reduce AKT phosphorylation (Figures 1 and 2 and data not shown)[15]. The minimum dose of caffeine to achieve a reduction in AKT activation appears to be 2.5 mmol/L, whereas the effective dose of pentoxifylline and theophylline ranges from 10-100 mmol/L.

Another important signaling pathway in the colon that can be activated by CHI3L1 is the β-catenin pathway. Stimulation of SW480 CECs using low dose of CHI3L1 results in an apparent β-catenin nuclear translocation[6]. In contrast to SW480 cells stimulated with CHI3L1 (80 ng/mL) that predominantly showed a nuclear localization of β-catenin, cells that were stimulated with CHI3L1 and concurrently treated with caffeine (5 mmol/L), but less significant with pentoxifylline (100 mmol/L) or theophylline (100 mmol/L), showed cytoplasmic β-catenin localization (Figure 3). Canonical activation of β-catenin requires the binding of the Wingless (Wnt) ligand onto the Frizzled receptor that subsequently stabilizes cytoplasmic β-catenin by destroying a protein complex (AXIN, GSK3β and APC) which usually cause the proteolysis of β-catenin under steady-state. This then facilitates the free β-catenin to migrate into the nucleus and subsequent activates transcription of target genes including *c-Myc* and *cyclin D1* (Figure 4). In a cohort study, high activation of β-catenin was found in 100%, 55% and 50% in IBD with colitis-associated cancer, IBD with dysplastic and IBD with remote dysplasia patients, respectively[61]. Recently, Lee *et al*[62] identified phosphatidylinositide 3-kinase (PI3K)/AKT signaling as the crucial factor mediating β-catenin during mucosal

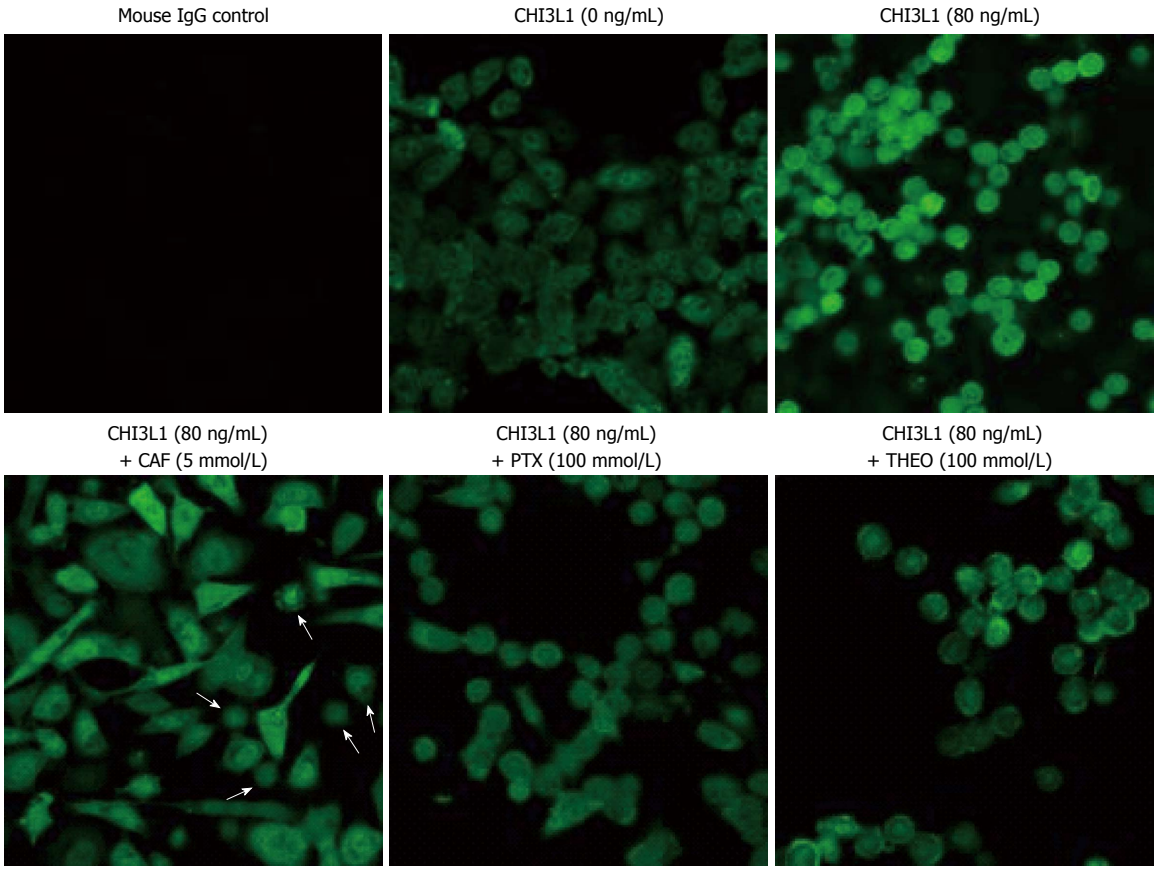

Figure 3 Caffeine, pentoxifylline and theophylline inhibit β-catenin nuclear translocation with different degrees. SW480 colonic epithelial cells were cultured on lab-tec chamber slide. After reached to 90% confluency, the cells were stimulated with or without purified human chitinase 3-like 1 (CHI3L1) (80 ng/mL) in combination with caffeine (CAF) (5 mmol/L), pentoxifylline (PTX) (100 mmol/L) or theophylline (THEO) (100 mmol/L) for 24 h. Human CHI3L1 protein was purchased from Quidel (San Diego, CA). β-catenin was then detected using mouse anti-human β-catenin monoclonal primary Ab (BD Biosciences, CA) and FITC-horse anti-mouse Immunoglobulin G (Vector Labs, Burlingame, CA) and analyzed by confocal microscope (magnification, objective 40 ×). White arrows show the limited numbers of completely nuclear translocated β-catenin positive cells after caffeine treatment.

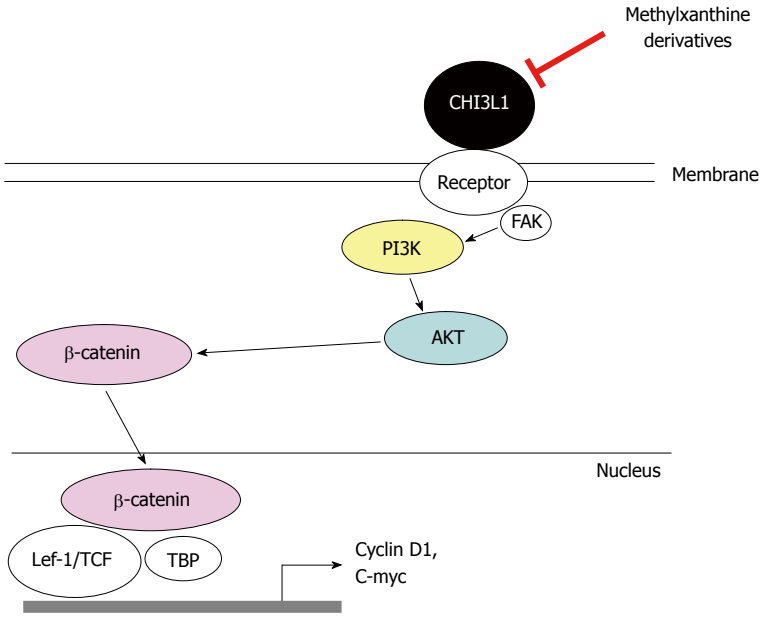

Figure 4 Schematic representation of chitinase 3-like 1-associated β-catenin activation signaling pathway, which is inhibited by methylxanthine derivatives. Binding of extracellular chitinase 3-like 1 (CHI3L1) to a putative receptor on plasma membrane activates the intracellular phosphatidylinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, which leads to β-catenin activation by translocating this protein from cytoplasm into nucleus. Methylxanthine derivatives, including caffeine, pentoxifylline and theophylline, directly down-regulate CHI3L1 mRNA expression and inhibit CHI3L1 protein functions, leading to reduced CHI3L1-associated AKT activation and prevent down-stream β-catenin nuclear translocation with different degrees of efficacy.

inflammation. They reported that IEC-specific PI3K conditional knockout mice showed reduced AKT and β-catenin signaling in the intestinal stem and progenitor cells and limits the extent of crypt epithelial proliferation. Inhibiting PI3K in IL-10 knockout mice, which develop spontaneous colitis, also impairs colitis-induced

epithelial AKT and β-catenin activation. Furthermore, a report by Fukumoto *et al*[63] also supports that viewpoint that AKT increases β-catenin activity by interfering with the AXIN/GSK3β complex. Chronic UC patients that have undergone 5-ASA treatment also show reduced AKT-mediated β-catenin phosphorylation in the middle and upper crypts in colon. This observation was recapitulated in 5-ASA treated IL-10 knockout colitic mice[64]. However, whether CHI3L1 mediated β-catenin activation is exerted directly through the Wnt or AKT pathway, or both but at different temporal time points or cell specific manner, remains to be investigated.

Currently, only a few receptors are known to bind to CHI3L1. Recently, He *et al*[65] identified that CHI3L1 binds to the interleukin-13 receptor α2 (IL-13Rα2) and activates both AKT and β-catenin signaling in the IL-13Rα2 dependent pathway. Therefore, exploring the use of methylxanthine derivatives for inhibiting CHI3L1 may block any downstream effects pertaining to AKT and/or β-catenin signaling and will provide direct mechanistic insights (Figure 4).

POTENTIALLY THERAPEUTIC/ PROPHYLACTIC EFFECTS OF METHYLXANTHINE-DERIVATIVES IN IBD

IN VIVO

Recently, our group performed in depth analysis of the role of caffeine treatment in a DSS-induced colitis model in mice[15]. Our *in vivo* analysis involved prophylactic-, simultaneous-, and post-treatment of mice with caffeine at 2.5 mmol/L in this animal model of intestinal epithelial damage. After initial caffeine treatment for 7 d, we challenged the mice with DSS in the drinking water for 5 d, and then returned to normal drinking water for 7 d before sacrificing. Mice which received the caffeine treatment protocol showed significantly improved symptoms as demonstrated by less percentage bodyweight loss and improved clinical scores. Colons of the mice were isolated, and it was shown that CHI3L1 and AMCase expressions were both significantly decreased after caffeine treatment. In contrast, chitinase 1 expression remained stable after the treatment. Colonic sections were also analyzed for histological changes. Mice in the caffeine-treated group demonstrated improved histological scores, with markedly decreased accumulation of immune cells, including $F4/80^+$, $CD4^+$, or $CD11b^+$ cells. Interestingly, bacterial colony forming units from homogenized mouse spleens, mesenteric lymph nodes, liver, cecum and colon were all significantly reduced after 2.5 mmol/L caffeine treatment. In addition, as we described in the previous section, the levels of several pro-inflammatory cytokines were significantly decreased in spleen, mesenteric lymph nodes, and colon, with an increase in the anti-inflammatory cytokine IL-10 in tissues. A major factor in IBD development is host-microbial interactions including adhesion/invasion of bacteria into the CECs and lamina propria. Caffeine treatment at both 2.5 and 5 mmol/L effectively prevented AIEC from invading into SW480 CECs, as well as in mouse-derived peritoneal macrophages. This result provides a possible explanation on therapeutic potential of caffeine in IBD through the prevention of CHI3L1-mediated bacterial adhesion/invasion.

In vivo testing using pentoxifylline to study the effects on IBD was also reported. Peterson *et al*[66] demonstrated that intra-rectal administration of pentoxifylline or 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (so called metabolite-1 or M-1) in a murine 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model, which showed an attenuation of colonic inflammation and intestinal fibrosis. M-1 is a chiral molecule derived from pentoxifylline by the reduction of a single ketone group to a corresponding hydroxy group. They reported that 64 mg/kg of pentoxifylline or M-1 is an ideal therapeutic dose in mice, whereas mice treated with 32 mg/kg showed varied effects in disease-associated phenotype. Another study also showed similar amelioration by pentoxifylline in TNBS-colitis in rats[67]. Interestingly, Murthy *et al*[68] showed that combining pentoxifylline with anti-TNFα antibody in DSS-induced colitic mice can reduce the side effects that is associated with anti-TNFα antibody treatment alone. *Ex vivo* studies also showed that peripheral mononuclear cells, which are obtained from the inflamed mucosa of CD and UC patients, reduce TNFα secretion by 50% in the presence of pentoxifylline (up to 100 μg/mL) for 24 h[69].

FUTURE OPTIMIZATION OF METHYLXANTHINE DERAVATIVES FOR IMPROVED SPECIFICITY AND EFFICACY INHIBITION OF FAMILY 18 CHITINASES

The early discovery of the allosamidin-derived from *Streptomyces* as a chitinase inhibitor has opened up opportunities to test the inhibitory effect on controlling chitinase-associated diseases[70]. For instance, as demonstrated in a study on AMCase-associated asthmatic Th2 inflammation mouse model, allosamidin, or anti-AMCase antibody, both independently can reduce bronchoalveolar lavage inflammation[71]. However, the concern over using allosamidin is its broad range of activity against all family 18 chitinases and less than ideal chemical properties (*e.g.*, high molecular weight and poor ligand efficiency)[72]. In addition, allosamidin has a stronger inhibitory effect on chitinase 1 than AMCase and therefore, since chitinase 1 is highly regarded as a molecule involved in host-defence system against a chitin-containing pathogen rather than a driver molecule involved in allergic inflammation, there is a need to identify or develop other chitinase inhibitor with higher specificity[73-75]. The discovery of the methylxanthine derivative inhibitory effects on family 18 chitinases appears to represent a promising alternative for its more suitable chemical properties and advantages as

described above sections. Yet, being a pan-chitinase inhibitor, it still faces a similar challenge in target specificity. Therefore, the next step is to optimize both specificity and efficacy of these methylxanthine derivatives.

In order to improve the inhibitory properties of the methylxanthine derivatives, Schüttelkopf *et al*[76] has developed a virtual algorithm method to create better family 18 chitinases. The algorithm, named as LIGTOR, basically fixed the methylxanthine substructure while performing torsional evaluation of the substitution based on previous published chitinase-pentoxifylline complex. Upon identification of the most desirable chemical features using this algorithm, the group then subsequently developed a low micromolar chitinase inhibitor that is composed of a two linked caffeine moieties that binds in the active site of the target extensively in a manner that was not previously reported. This di-caffeine compound, subsequently named as bisdionin B, showed the desired drug-like structure, as demonstrated by X-ray crystal structure analysis, and provides a general scaffold for future development/optimization of the family 18 chitinase inhibitors.

Another major concern in drug design is target specificity. As a pan-chitinase inhibitor, one of the major drawbacks of methylxanthine derivatives is the discrimination between the different chitinases (*e.g.*, CHI3L1, AMCase and chitinase 1). To address this issue, Sutherland *et al*[77], utilized the LIGTOR algorithm derived di-caffeine scaffold and modified the caffeine linker length and subsequently analysed it against the AMCase crystal structure. They then developed a derivative of the di-caffeine scaffold, termed as bisdionin F, that showed a high selectivity for human AMCase up to 20-fold over chitinase 1. The exact orientation/coordinates were confirmed by crystal structure of the human AMCase-bisdionin F complex. The group further validated the efficacy of bisdionin F in a murine model of allergic inflammation. All these suggest that further improvements can be made to develop a molecule with improved inhibitory efficacy and higher specificity against the targeted molecule of the chitinase 18 family.

CONCLUSION

CHI3L1 is an important inducible molecule on IECs and acively participates in the pathogenesis of chronic inflammation and inflammation-associated malignant transformation of epithelial cells. Methylxanthine derivatives, including caffeine, theophylline and pentoxifylline, can potentially suppress inflammation *via* CHI3L1 inhibition. The result in this study may provide the conceptual framework for a new class of therapeutic agents, which will effectively prevent chronic inflammatory diseases with minimal side effects.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

When combination therapy isn't working: Emerging therapies for the management of inflammatory bowel disease

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Abstract

Although antagonists of tumor necrosis factor have resulted in major therapeutic benefits in inflammatory bowel disease, the magnitude and durability of response are variable. Similar to previously available drugs such as 5-aminosalicylates and immunomodulators, the therapeutic effect is not universal leaving many people searching for options. The development of newer agents has benefited from advances in the understanding of the pathophysiology of the disease. Uncontrolled activation of the acquired immune system has an important role, and lymphocytes, cytokines, and adhesion molecules are broadly targeted for therapeutic intervention. There is increasing evidence of an important role of the innate immune system and the intestinal epithelium, and the therapeutic paradigm is also shifting from immunosuppression to the reinforcement of the intestinal barrier, and modification of the disease process. In this review, we explore the limitation of current therapy as well as mechanisms of actions of new drugs and the efficacy and adverse events from data from clinical trials.

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Key words: Inflammatory bowel disease; Emerging therapies; Vedolizumab; Ustekinumab; Tofacitinib

Core tip: In this paper we critically review recently published literature about these novel therapies, which have been the results of extensive research identifying molecular targets. Several agents have been tested and show promising data, but we focus on vedolizumab, a monoclonal antibody against the $\alpha 4\beta 7$ integrin on lymphocytes, ustekinumab, a monoclonal antibody against the p40 subunit of interleukin-12 and interleukin-23, and tofacitinib, an orally administered small molecule targeting Janus-activated kinase. These three agents are most likely to find their way soon to the market and offer significant therapeutic advantages for the management of Crohn's disease and ulcerative colitis.

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INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are thought to be the result of an overly aggressive immune response toward an environmental trigger within a genetically susceptible host. Recent work has elucidated the host-microbe interaction identifying the complex interplay between genetic susceptibility, environmental factors, and the intestinal microbiome[1]. This has led to a deeper understanding of the immunologic pathways leading to IBD and, most importantly, to the development of targeted therapies. For CD and UC not responding to 5-aminosalicylates (5-ASA), immunosuppression with corticosteroids, aza-

thioprine, and anti-tumour necrosis factor (anti-TNF) antibodies have been the mainstay of treatment[2]. Even the most potent combination of immunomodulator and anti-TNF therapy in combination in recently diagnosed CD only achieves steroid free remission in 57% of patients[3]. Given the persistent and sizable population of patients who are not served with current therapy, there has been great interest in new pathways of inflammation that would be amenable to pharmacologic intervention. This paper focuses on the most promising pathways and medications that appear closest to clinical availability.

LIMITATIONS OF CONVENTIONAL THERAPY

Corticosteroids

The significant benefit of corticosteroid therapy in IBD was established in the 1950s and 1960s for UC and later in the 1970s and 1980s for CD[4]. In general, corticosteroids centrally suppress nuclear factor (NF)-κB activation, which is the primary transcription factor mediating inflammatory response in both the innate and adaptive immune systems. In active IBD, corticosteroids are still a viable first-line treatment, but patients and clinicians have to be aware of significant short- and long-term side effects. Opportunistic infections, steroid-induced psychosis, steroid dependence, diabetes, and osteoporosis are among the most common side effects[5]. Clinicians should also be aware of the variability of clinical response to these agents. Corticosteroids are unlikely to induce mucosal healing or maintain clinical remission, so its use is limited to induction of remission[6].

Thiopurines

Thiopurines, also referred to as immunomodulators, are derivatives of thioguanine and act as purine antimetabolites. Following metabolization into 6-thioguanine nucleotides, immunosuppression is in part the result of incorporation into the DNA/RNA of rapidly dividing inflammatory cell lines[7]. This induces effector T cell apoptosis by suppression of the Rac1 and Vav-Rac1 signaling pathways and decreases NF-κB activation, which leads to a decrease in pro-inflammatory cytokine secretion. Up to one third of patients with IBD are intolerant to thiopurines and a further 10% are unresponsive to them[7]. In the majority of patients who do respond, the benefits of thiopurines take 3-6 mo to appear[8]. Significant risks of thiopurines include lymphoproliferative disease (non-Hodgkin's lymphoma), as high as 4-5 fold compared with unexposed IBD patients and further increased when used in combination with anti-TNF[9]. Other side effects include early hypersensitivity reactions such as fever and pancreatitis, bone marrow suppression and hepatotoxicity requiring frequent lab monitoring during treatment[10]. Finally, one in 300 patients harbor a homozygous *TPMT* mutation and use of these agents is generally avoided[11].

Methotrexate

For patients with CD unresponsive to thiopurines, methotrexate has been the alternative. In a recent study from 2011, Kozarek *et al*[12] showed that in patients who had failed azathioprine treatment, methotrexate was effective in maintaining clinical benefits in 63% of patients at 1 year. And in this group, 26% required side effects sufficient enough to discontinue therapy, and most of these adverse events occurred in the first 6 mo. This study shows that methotrexate is well tolerated in the long term and is a viable option in patients who cannot receive thiopurines. However, methotrexate has lower mucosal healing rates compared to biologics and azathioprine[12].

Methotrexate is an antimetabolite and acts specifically during DNA and RNA synthesis and therefore its effect is mainly seen on rapidly dividing cells (such as gastrointestinal and oral mucosa, and effector T-cells). This mechanism of action explains the most frequently observed side effects of myelosuppression and liver. Furthermore, its absolute contraindication in pregnancy makes this drug less desirable to use in reproductive aged women[13]. Unlike, thiopurines methotrexate is not effective in inducing or maintaining remission in UC[14]. In addition, a significant percentage of prescribing gastroenterologists were unfamiliar and uncomfortable with its use in the management of CD, further limiting its use in the United States[15].

Biologic therapy

Anti-TNF-α inhibitor therapy: TNF-α is a key pro-inflammatory cytokine involved in the systemic inflammatory cascade and is a member of a group of cytokines that stimulate the acute phase reaction. In response to lipopolysaccharide and other bacterial products presented by antigen-presenting cells, activated intestinal T cells, macrophages, produce TNF-α and natural killer cells. In IBD, the number of TNF-α secreting T cells in the lamina propria is increased and specific agents directed at these T cells were developed[16].

The United States Food and Drug Administration (FDA) approved the first anti-TNF agent for CD, infliximab, in 1998. This was followed by additional in category drugs, adalimumab in 2007, which had the benefit of subcutaneous administration, and certolizumab in 2008.

While various trials have confirmed the significant clinical benefit of anti-TNF-α therapy, it is apparent that there are major limitations in the use of these agents, ranging from cost-effectiveness issues to morbidity and mortality, and the side effect profile is significant[17]. Data from large treatment registries suggest that patients receiving both anti-TNF-α agents and other immunosuppressants such as azathioprine and corticosteroids are at a higher risk of developing opportunistic infections and lymphoma[9]. In addition, approximately 10% per year of patients lose response to this therapy, often but due to the development of anti-drug antibodies[18,19].

Therapies targeting other cytokine pathways

Natalizumab: Natalizumab, a human anti-α4 integrin

antibody, has been studied in CD[20] and a recent meta-analysis concluded that it was superior to placebo in controlling symptoms and inducing mucosal healing[16]. However, its adoption was limited by the discovery of the development of progressive multifocal leukoencephalopathy in 1:1000 patients[21]. After initially being removed from the market, it's currently available only through enrollment in a specialized FDA risk minimazation program known as TOUCH (https://www.touchprogram.com/TTP) that details the risk of progressive multifocal leukoencephalopathy (PML).

New therapeutic goals: Mucosal healing and deep remission: In recent years, mucosal healing has emerged as a major therapeutic goal in IBD. However, the definition of mucosal healing varies across studies, and there is no validated definition of mucosal healing or endoscopic remission in IBD[22]. Current therapeutic goals are to induce remission both clinically, biochemically and endoscopically[23].

"Deep remission" in CD is defined as a combination of endoscopic and clinical indicators of disease activity, a CD activity index (CDAI) below 150 together with complete mucosal healing. Deep remission has been associated with better long term disease-specific quality of life, fewer flares and Crohn's related hospitalizations, greater work productivity, and less activity impairment compared to mucosal healing alone[24]. In UC, there is no proposed definition of deep remission.

Another area of consensus regarding potential therapy is prevention of bowel damage in CD by earlier introduction potent of therapy. In a population-based cohort study from Olmsted County, United States, among patients with CD diagnosed from 1970 to 2004, the cumulative risk of developing complications (structuring or penetrating disease) was 34% at 5 years and 51% at 20 years after diagnosis[25]. These observations highlight the importance of creating tools that are able to measure cumulative bowel damage in CD. The development of an appropriate index is underway and has been named the CD digestive damage score (the Lemann score)[26]. It addresses damage location, severity, extent, progression and reversibility, as measured by diagnostic imaging modalities and the history of surgical resection. The Lemann score may be used to assess the effect of various pharmacological therapies, function as a clinical trial endpoint and allow better identification of high-risk patients in regard to identification or progression of bowel damage.

The concept of early treatment to avoid later complications and the need for surgical intervention in CD is gaining momentum, and these new scoring systems might be able to help to identify patients at risk and help caregivers determine the timing for introduction of disease modifying agents. In a population-based study from Cardiff, United Kingdom, Ramadas *et al*[27] reported that early thiopurine use (within the first year of diagnosis) was associated with lower rates of surgery. Subgroup analysis from placebo-controlled trials with anti-TNF-α agents have also suggested that patients with early CD may experience greater efficacy than patients with established disease[28].

Complicated CD can be defined as the presence of bowel damage (stricture, abscess and/or fistula) and/or the requirement for surgery. The clinical factors associated with complex CD include: ileal disease, upper gastrointestinal involvement, smoking, complicated behavior (stricturing or penetrating), young age at diagnosis, perianal disease and some genetic factors such as nucleotide oligomerization domain 2 (*NOD2*), interleukin 10 (*IL10*) or *IL-10R* mutations[29]. The most widely studied genetic marker in CD is the presence of variants of the *NOD2* gene and its association with a more complex disease course and requirement for surgeries[30]. The IBD CHIP project, a new DNA array-based diagnostic system which uses a DNA array to detect IBD mutation to predict the clinical evolution for CD, found that the *NOD2* gene was the most important genetic association, was an independent predictive factor for need for surgery, and the strongest factor associated with a complicated disease course. However, a more recent study has reported that associations with gene variants are not accurate in predicting the course of CD. Currently, serological and genetic markers are not routinely used in clinical practice, as data are variable in the predictive value of these markers[31-33].

Aggressive UC was recently defined as disease that is associated with a high relapse rate, the need for surgery, the development of colon cancer and/or the presence of extra-intestinal manifestations[34], though this marks a very heterogeneous group of patients. Clinical risk factors include extensive colitis and a young age at diagnosis. As in trials for rheumatoid arthritis, new emphasis is being placed on disease modifying drugs which limit mucosal damage. This marker of therapy may be used in upcoming disease-modification trials as well as in our clinical practice to promote early intervention with disease modifying agents in patients having such factors[35].

Emerging concepts in the management of IBD patients are tight monitoring and accelerated step up approaches[36]. These require endoscopy, imaging (magnetic resonance imaging) and colonoscopy, C reactive protein, and/or fecal markers at 3-6 mo after introduction of disease-modifying agents (thiopurines and anti-TNF-α) to identify objective signs of inflammation, and escalation of treatment. Further clinical trials are needed to evaluate the value of this strategy.

In CD patients with mild disease, corticosteroids are appropriate on an as needed basis, and in patients with moderate active disease without poor prognostic indicators, steroids and thiopurines still remain the standard first-line therapy. Anti-TNF–α therapy should be considered as first-line therapy in patients with CD with bowel damage (stricture/fistula/abscess) and/or poor prognostic factors, severe disease, or complex perianal disease[33,36].

In UC, a proposed treatment algorithm for acceler-

ated step-up therapy after "5-ASA failure" indicates that steroids and azathioprine should be considered. If in case of is steroid-dependent disease and persistent signs of inflammation, then long term maintenance therapy with thiopurines or anti-TNF-α agents should be considered[34,37].

Despite therapeutic advances over the past several years and introduction of multiple new anti-TNF agents, including infliximab, adalimumab, certolizumab, golimumab, and natalizumab (CD only), there is still a large subset of patients that do not respond to these drugs or are unable to maintain remission long-term[38-41]. It is likely that these patients have disease that is driven by other factors. The best management approach toward these patients is unclear, but the development of new non anti-TNF based therapies may be a promising avenue of treatment. Three new agents in the "pipeline" which appear to be promising, and are closest to being commercially available in the United States are ustekinumab, tofacitinib, and vedolizumab. Ustekinumab already has FDA approval for psoriasis, and tofacitinib has FDA approval for rheumatoid arthritis, vedolizumab will soon be evaluated by the FDA for approval as IBD therapy[35], and is expected to be approved in 2014.

Targeting IL-12/23: IL-12 and IL-23 are inflammatory cytokines produced by antigen-presenting cells, which promote T cell maturation into T-helper (Th)1 and Th17 phenotypes, respectively. The cytokines were identified to be significant to the pathology IBD in genome wide association studies[42]. IL-12 and IL-23 share a common p40 subunit, and it is known that when IL-12 (p35 + p40) is present, T cells differentiate into Th1 cells producing interferon-γ and TNF. On the other hand, when IL-23 (p19 + p40) is present together with transforming growth factor-β and IL-6, the Th17 subset preferentially develops and produces IL-6, IL-17A, IL-17F, IL-22 and IL-21[35]. Since both pathways are activated in CD patients, and contribute to tissue damage by the production of inflammatory cytokines, this makes neutralizations of the p40 subunit an attractive therapeutic target. Ustekinumab (Stelara) and briakinumab (Ozespa, previously ABT-874; Abbott, Abbot Park, IL, United States) are monoclonal IgG1 antibodies that target the p40 subunit of the IL-12/IL-23. Although Phase Ⅱ trials in briakinumab were negative, however ustekinumab showed promising results in CD and is being further evaluated[43,44].

The efficacy of ustekinumab was initially investigated in a double-blind, cross-over trial with 104 moderate to severe CD patients. In this group, clinical response rates for the patients given ustekinumab and placebo were 53% and 30%, respectively (P = 0.02) at weeks 4 and 6, and 49% and 40% (P = 0.34) at week 8. Further subgroup analysis showed that patients who received infliximab in the past (neither primary nor secondary non responders) had a significantly greater response to ustekinumab (P < 0.05) through week 8. Based on the results of this study it was noted that ustekinumab induced clinical response in patients with moderate to severe disease, especially in those with prior exposure to infliximab. These results led to further evaluation of ustekinumab in inducing and maintaining remission in patients with CD refractory to anti-TNF agents. During induction, 526 patients were randomly assigned to receive intravenous ustekinumab (at dose 1, 3, or 6 mg/kg of body weight) or placebo at week 0. In the maintenance phase, the 145 patients who had a response to ustekinumab at 6 wk underwent a second randomization to receive subcutaneous ustekinumab (90 mg) or placebo at weeks 8 and 16, the primary endpoint was clinical response at week 6 defined as decrease in CDAI of 100 points from baseline. The proportions of patients who met the primary endpoint were 36.6%, 34.1%, 39.7% for 1, 3 and 6 mg of ustekinumab, respectively *vs* 23.5% for placebo (P = 0.005 for 6 mg group). The proportion of patients who achieved clinical remission was not significantly different between any of the groups at week 6. However, maintenance therapy with ustekinumab, as compared with placebo, resulted in significantly increased rates of response (69.4% *vs* 42.5%, P < 0.001) and clinical remission (41.7% *vs* 27.4%, P = 0.03) at 22 wk. Serious infections occurred in 7 patients (6 receiving ustekinumab) during induction and 11 patients (4 receiving ustekinumab) during maintenance therapy[43,45] (Table 1).

We await the results of an ongoing phase 3 study. If the data are confirmed, this therapy may become a useful option for patients who have failed anti-TNF therapy.

ANTIADHESION MOLECULES

Drugs targeting adhesion molecules interfere with the migration of leukocyte subsets from the blood to the sites of inflammation[46]. The first drug in this category to be used in IBD was a monoclonal antibody against the α4 integrin, natalizumab. The blockade of α4-integrins not only interferes with the α4β7 MadCam1 interaction, associated with IBD, but also with α4β1 vascular cell adhesion molecule-1 interaction which is needed for patrolling effector T cells to contain JC virus and prevent it from infecting the brain. Following these results, therapies targeting α4β7 more specifically for the gut vasculature were developed in order to avoid the risk of PML[47].

Vedolizumab (formerly called MLN02, Millenium; Takeda) binds specifically to the α4β7-integrin dimer which is involved in recruitment of lymphocytes to the gut. Two large phase 3 studies under the GEMINI study group, one in UC (GEMINI 1) and one in CD (GEMINI 2) have recently been published and demonstrate a beneficial effect of vedolizumab in induction and maintenance of remission of UC and CD. GEMINI 1 and 2 are randomized, blinded, placebo-controlled multicenter trails to examine the efficacy of vedolizumab for induction and maintenance in moderate to severe UC and CD, respectively.

In the induction trial of the GEMINI 1 study, 374 patients received vedolizumab or placebo at week 0 and

Table 1 Clinical response defined as decrease in Crohn's disease activity index > 100

Therapeutic agent	Disease tested	Response *vs* placebo	Target patients	Ref.
Ustekinumab	Crohn's disease	Clinical response: 69.4% *vs* 42.5%, $P < 0.001$ Remission: 41.7% *vs* 27.4%, $P = 0.03$	Adult patients with moderate to severe Crohn's disease with failure to anti-tumour necrosis factor (TNF) therapy	[43]
Vedolizumab	Crohn's disease	Clinical response: 31.4% *vs* 25.6%, $P = 0.23$ Remission at week 52: 14.5% *vs* 6.8%, $P = 0.02$	Adult patients with active Crohn's disease (Crohn's disease activity index, 220-450) with previously documented lack of response to glucocorticoids or immunosuppressive agents or anti-TNF agents	[52,53]
	Ulcerative colitis	Clinical response: 47.1% *vs* 25.5%, $P < 0.001$ Remission at week 52: 41.8%, 44.8% (8 and 4 wk dosing interval, respectively) *vs* 15.9%, $P < 0.001$	Adult patients with active ulcerative colitis (Mayo score 6 to 12) with disease at least 15 cm proximal from anal verge, with previously documented unsuccessful treatment with glucocorticoids or immunosuppressive medications or anti-TNF	
Tofacitinib	Ulcerative colitis	Clinical response: 32%, 48%, 61%, 78% [at dose 0.5 mg ($P = 0.39$), 3 mg ($P = 0.55$), 10 mg ($P = 0.10$), and 15 mg ($P < 0.001$)] *vs* 42% for placebo Clinical remission: 13%, 33%, 48%, 41% [at dose 0.5 mg ($P = 0.76$), 3 mg ($P = 0.01$), 10 mg ($P < 0.001$), and 15 mg ($P < 0.001$)], *vs* 10% for placebo	Adults patients with moderate to severe ulcerative colitis	[52]

2, and 521 patients received open-label vedolizumab at weeks 0 and 2 with disease evaluation at week 6. Patients who had a response at week 6 were then randomly assigned to continue receiving vedolizumab every 8 or 4 wk or were switched to placebo for up to 52 wk. The primary outcome was clinical response at week 6 defined at a reduction in the Mayo score of at least 3 points and a decrease of at least 30% from the baseline score. Response rates at week 6 were 47.1% and 25.5% among patients in the vedolizumab group and the placebo group, respectively ($P < 0.001$). At week 52, 41.8% of patients who continued to receive vedolizumab every 8 wk and 44.8% of patients who received vedolizumab every 4 wk were in clinical remission, as compared with 15.9% of patients who switched to placebo ($P < 0.001$ for both groups). The frequency of side effects was similar in both groups and serious adverse events were not more common in the vedolizumab group. All patients in the trial had an eligibility criterion of an unsuccessful previous treatment (lack of response, or unacceptable side effects), with one or more of the following medications: glucocorticoids, immunosuppressive medications (azathioprine or 6-mercaptopurine), or TNF antagonists. Patients were allowed to continue 5-ASA drugs during the study. This study shows that vedolizumab is more effective than placebo as induction and maintenance therapy in UC and more importantly, shows response in patients who had failed previous therapy[48,49].

In the induction trial for GEMINI 2, 368 patients were randomly assigned to receive vedolizumab or placebo at weeks 0 and 2 and 747 patients received open label vedolizumab at weeks 0 and 2; disease status was assessed at week 6, and the two primary endpoints were clinical remission (CDAI < 150) and CDAI-100 response (> 100 point decrease in CDAI score) at week 6. In the maintenance trial, 461 patients who had had a response to vedolizumab were re-randomized to placebo or vedolizumab every 8 or 4 wk until week 52. At week 6, a total of 14.5% of patients who received vedolizumab were in clinical remission, compared to 6.8% of patients who received placebo ($P = 0.02$). A total of 31.4% and 25.7% of patients in the treatment *vs* placebo group had a CDAI-100 response ($P = 0.23$). Among the 461 patients who had an initial response, 39.0% and 36.4% of those assigned to vedolizumab every 8 or 4 wk were in clinical remission at week 52, compared to 21.6% of those assigned to placebo ($P < 0.001$ and $P = 0.004$, for the two vedolizumab groups, respectively). Vedolizumab, as compared with placebo, was associated with a higher rate of serious adverse events (24.4% *vs* 15.3%), infections (44.1% *vs* 40.2%), and serious infections (5.5% *vs* 3.0%). Eligible patients for the trial had had no response to or had had an unacceptable side effects from one or more of the following: glucocorticoids, immunosuppressive agents (azathioprine, 6-mercaptopurine, or methotrexate), or TNF antagonists. This study shows that vedolizumab-treated patients with active CD were more likely than patients receiving placebo to have a remission, but not a CDAI-100 response, at week 6 (primary endpoint); and that those patients with an initial clinical response were more likely to be in clinical remission at week 52[50].

Both these reports are among the largest clinical studies in patients with IBD and combined consist of 2010 patients. Primary and secondary endpoints were met in the GEMINI 1 study (UC), however it seems that vedolizumab was less effective in patients with CD, although maintenance of remission was noted in the treatment group, and that patients with CD had a higher frequency of adverse events with treatment compared to placebo. It is possible that the underlying pathogenesis of the two diseases are different, and the selective blockage of gut-specific $\alpha 1\beta 4$-mediated leukocyte trafficking is more beneficial in UC, which is confined to the mucosa and the large intestine, compared to CD which might repre-

sent a more systemic disorder. More studies need to be done to assess the pharmacodynamics of vedolizumab. To date, there have been no reported cases of PML. The results of these two recent studies suggest a promising new therapy for IBD.

TARGETING JANUS KINASE RECEPTORS

Besides specific neutralization of specific cytokines with antibodies, control of inflammation can be achieved by interfering with conserved elements associated with cytokine receptors, thus allowing a broader action. The Janus kinase (JAK) family of tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) plays a significant role in signal transduction of hematopoietin receptors type I and II. Tofacitinib (formerly CP-690, 550; Pfizer) is a JAK inhibitor that inhibits all four JAK family kinase members, with a functional cellular specificity for JAK1 and JAK3 over JAK2. Thus it can directly or indirectly modulate signaling for an important subset of proinflammatory cytokines including IL-2, -4, -7, -9, -15 and -21[51].

In a double-blind, placebo controlled, phase 2 trial, 194 patients with moderately to severely active UC were randomized to receive tofacitinib at a dose of 0.5, 3, 10 or 15 mg or placebo twice daily for 8 wk. Patients enrolled had to have a Mayo score of at least 6 with an endoscopic subscore of 2 or 3, and patients could receive oral mesalamine or oral prednisone at a stable dose of 30 mg or less per day, patients were not allowed to be on concurrent immunosuppressive therapy or therapy with anti-TNF agents[36]. Approximately 30% of patients had had prior anti-TNF exposure with failure of therapy. The primary outcome was clinical response at 8 wk, and occurred in 32%, 48%, 61% and 78% of patients receiving tofacitinib at a dose of 0.5 mg ($P = 0.39$), 3 mg ($P = 0.55$), 10 mg ($P = 0.10$) and 15 mg ($P < 0.001$), respectively, compared with 42% of patients receiving placebo. Clinical remission, defined as Mayo score < 2 with no subscore > 1, at 8 wk occurred in 13%, 33%, 48%, and 41% of patients receiving tofacitinib at a dose of 0.5 mg ($P = 0.76$), 3 mg ($P = 0.01$), 10 mg ($P < 0.001$) and 15 mg ($P < 0.001$), respectively, compared with 10% of patients receiving placebo. The most commonly reported adverse events was related to infection, and occurred in 6 patients on treatment with any dose of tofacitinib and 6 patients in the placebo group. There was a dose dependent increase in both high and low density lipoprotein cholesterol at 8 wk with tofacitinib which reversed after discontinuation of the study drug[52]. Given the broad mechanism of action, opportunistic infections remain a valid concern and more data is required to determine the efficacy and safety of tofacitinib in the treatment of IBD. However, it's oral method of administration will surely make this a popular avenue of treatment should it prove effective in larger scale treatment trials (Table 1).

CONCLUSION

Although IBD therapy has become much more efficacious with the introduction of anti-TNFs and the use of combination therapy, many patients still experience insufficient improvement on these agents. We are currently limited in option for patients who fail to respond to anti-TNF agents. In the near future, our patients may have access to IL-12/23 antibodies in CD, vedolizumab in CD and UC, possibly tofacitinib in both UC and CD. The promise of these agents is a bright light on the horizon for treatment of IBD.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Prevention of post-operative recurrence of Crohn's disease

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Abstract

Endoscopic and clinical recurrence of Crohn's disease (CD) is a common occurrence after surgical resection. Smokers, those with perforating disease, and those with myenteric plexitis are all at higher risk of recurrence. A number of medical therapies have been shown to reduce this risk in clinical trials. Metronidazole, thiopurines and anti-tumour necrosis factors (TNFs) are all effective in reducing the risk of endoscopic or clinical recurrence of CD. Since these are preventative agents, the benefits of prophylaxis need to be weighed-against the risk of adverse events from, and costs of, therapy. Patients who are high risk for post-operative recurrence should be considered for early medical prophylaxis with an anti-TNF. Patients who have few to no risk factors are likely best served by a three-month course of antibiotics followed by tailored therapy based on endoscopy at one year. Clinical recurrence rates are variable, and methods to stratify patients into high and low risk populations combined with prophylaxis tailored to endoscopic recurrence would be an effective strategy in treating these patients.

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Key words: Inflammatory bowel disease; Crohn's disease; Postoperative recurrence; Medical treatment; Biologics

Core tip: This review summarizes and updates the current state of the field of post-operative prevention of Crohn's disease (CD) after surgery. This review starts by discussing the natural history of postoperative recurrence followed by a summary of the most consistent and evidence based risk factors. We then discuss the evidence for medical prophylaxis of CD highlighting new data regarding biologics. Finally we discuss cost effectiveness and provide a potential novel treatment algorithm for a clinician to use practically when caring for a patient with CD after surgery.

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INTRODUCTION

Patients with Crohn's disease (CD) have a high likelihood of requiring surgery over their lifetime. Reasons for surgical intervention include failure of medical therapy, or a complication of stricturing or penetrating disease[1]. It is estimated that half of patients will require surgery within 10 years of diagnosis[2,3] and around 80% of patients will require surgery at some time in their life[4-6]. Unfortunately, surgery for CD is not curative and recurrence is typically the rule. Symptomatic recurrence varies from 40%-80%[5,7]. Endoscopic recurrence is much higher with up to 90% having lesions at 5 years[4,7,8].

While the overall recurrence rates are high, the severity of recurrence varies amongst individuals. Some patients

may only develop mild endoscopic recurrence, and have no symptoms, whereas others may present soon after surgery with recurrent inflammation and clinical symptoms. Interventional studies have measured a drugs ability to prevent endoscopically-visible ulcers, or radiologically-apparent luminal narrowing, or symptoms. Assessing for severe endoscopic recurrence is useful, although still a surrogate predictor of future clinical recurrence. The wide variation of patients and outcomes used in clinical trials makes this topic difficult for the treating physician to apply to a given patient. The aim of this review is to summarize the literature regarding the medications available for prevention of post-operative recurrence as well as to discuss the different strategies for treating postoperative recurrence.

NATURAL HISTORY OF POST-OPERATIVE CROHN'S

The natural history of CD generally follows phenotypic patterns, whether in the pre-operative or post-operative patient. As stated above, recurrence in general is common both endoscopically and symptomatically after surgery. Crohn's recurrence after ileocecal resection or colectomy with ileorectal anastomosis tends to favor the anastomosis site, specifically the small bowel proximal to the anastomosis[9]. Initially aphthous ulcers develop which then progress to larger ulcers and eventually complications of recurrent CD[7,8]. It was noted in the late 1980s that patients who underwent surgery for a perforating phenotype of disease had a different post-operative course than those who underwent surgery for non-perforating indications[10]. This group examined 770 patients who underwent surgery for CD at Mt. Sinai Hospital in New York. They found that not only did patients had a different course depending on their pre-operative phenotype, but they found that patients who required second and third operations, tended to do so for the same initial indication. Thus, patients who required an initial operation for penetrating disease tended to require a second, and potentially third, operation for penetrating disease. Thus overall the site and prevailing phenotype are thought to be similar before and after resection[11].

An interesting observation into the pathogenesis of post-operative CD is that the fecal stream is needed for recurrence. Rutgeerts *et al*[8] reported on five patients who underwent ileal resection with ileocolonic anastomosis and additionally had a temporary, diverting ileostomy approximately 25-30 cm from their anastomosis site. At six months time, the anastomoses of these patients were compared to 75 other patients who underwent ileocolonic anastomosis without diverting ileostomy. None of the patients with the diverting ileostomy developed endoscopic or histologic changes consistent with CD compared to 71% of patients in the control group who had endoscopic recurrence at six months. All five patients however, did develop endoscopic recurrence of disease at the anastomosis six months after the diverting ileostomy was taken down[12]. Further study demonstrated that contact with intestinal fluid for only eight days was sufficient to trigger early histologic changes in a previously normal neo-terminal ileum and anastomosis[13]. Within three months after ileocecectomy the neoterminal ileum has been shown to have an increase in colonization with colonic flora, specifically *Escherichia coli* (*E. coli*), *Enterococci*, *Bacteroides* and *Fusobacteria* spp[14]. Animal studies have also concluded that the presence of commensal bacteria is required for post-operative recurrence to develop[15]. It remains unclear if these changes have a causative role in post-operative Crohn's recurrence, but they have been implicated in IBD *per se*[16]. Thus, post-operative CD is associated with alterations in the local microbiome, development of local ulcers (endoscopic recurrence) and subsequent resumption of clinical recurrence.

RISK FACTORS FOR RECURRENCE: RISK STRATIFICATION

Multiple risk factors have been identified for postoperative recurrence. Risk stratifying patients based on these factors can thus help decide which patients to treat aggressively. Unfortunately, few risk factors have been consistent in the literature for predicting endoscopic, clinical or surgical recurrence. Typically risk factors are divided into patient-related factors, disease-related factors and surgical-related factors. Of the patient related factors, only smoking has consistently been identified as a predictor of postoperative recurrence. The odds ratio for clinical recurrence for current smokers is between 2 and 3[4,17,18]. It is the only modifiable risk factor and smoking cessation appears to decrease the risk of recurrence to that of a non-smoker[19]. Other patient related factors such as age, sex, age at disease onset are inconsistent risk factors in the literature[20,21].

Disease related factors include duration of disease prior to first resection, history of previous resection and penetrating disease[4,22]. Duration of disease prior to first surgery is not a consistent risk factor in the literature[23,24]. Most studies demonstrate that previous surgery for CD is a risk factor for further surgery[11,22,25]. A recent meta-analysis demonstrated a hazard ratio of 1.5 for recurrence of disease for penetrating phenotype compared to non-penetrating[26]. Unfortunately, there was significant heterogeneity in the studies that went into the meta-analysis and thus it is not entirely clear what the relationship between penetrating disease and post-operative recurrence truly is[21]. Further complicating the issue is that penetrating and stricturing disease are not always accurately diagnosed on clinical grounds. Up to a third of patients who clinically have non-penetrating disease may have evidence of penetration at the time of an operation[27].

Surgical factors including length of resection, type of anastomosis and histological disease left in the bowel or granulomas in the resected specimen have not been

Table 1 Risk factors for postoperative recurrence

Risk factor	Strength of association (OR)	Grade of evidence[1]
Active smoking	2 (1.3-3.4)[18]	B
Penetrating disease	1.5 (1.2-1.9)[26]	B
Prior resection	1.8 (1.1-2.9)[22]	B
Myenteric plexitis	1.9 (1.0-3.5)[33]	C

[1]Based on American Gastroenterological Association guidelines. A: Multiple, well-designed randomized controlled trials; B: At least one large well-designed clinical trial with or without randomization; C: Based on clinical experience, expert committees or descriptive studies.

consistently demonstrated to be risk factors for disease recurrence[28-32]. However, myenteric plexitis in the resection specimen has been found to be predictive of post-operative recurrence[33-35]. One small study found plexitis to be associated with prior intestinal surgery and shorter duration of disease thus potentially accounting for some of the variability of those risk factors as predictors of disease[36]. Select risk factors that have been consistent in the literature are shown in Table 1.

MEDICAL THERAPIES TO PREVENT RECURRENCE

Multiple medications used for the treatment of Crohn's disease have been studied for the prevention of post-operative recurrence. Table 2 summarizes the endoscopic and clinical recurrences rates *vs* placebo from various trials.

5-ASA

The use of mesalamine in the postoperative setting is appealing given its favorable safety profile, ease of administration, and relatively lower costs to anti-TNFs. However controversy exists over its efficacy. McLeod *et al*[31] demonstrated a 10% decrease in symptomatic recurrence (P = 0.03) with mesalamine at 3.0 g per day. Others have noted a decrease in endoscopic recurrence with varying mesalamine formulations[37,38]. Despite this, some small prospective and retrospective studies failed demonstrate a difference compared to placebo[39,40]. Interestingly, Hanauer *et al*[41] found that postoperative mesalamine demonstrated a non-significant trend towards a clinical improvement compared to placebo, but did not have any effect on endoscopic or radiologic recurrence. A meta-analysis of available prospective studies did demonstrate that mesalamine decreased clinical as well as severe endoscopic recurrence at 12 mo with a NNT of 8 (RR *vs* placebo: 0.76, 95%CI: 0.62-0.94)[42].

Thiopurines

Azathioprine and 6-mercaptopurine are efficacious in maintenance of CD and have been extensively studied in the post-operative setting. Only two studies have compared to a thiopurine to placebo and one was in combination with three months of metronidazole[41,43]. Both trials showed a decrease of endoscopic recurrence in the thiopurine arm at 12 mo. Pooling the data demonstrated a favorable risk ratio for decreasing both clinical (RR *vs* placebo: 0.59, 95%CI: 0.38-0.92) and severe endoscopic recurrence (RR *vs* placebo: 0.64, 95%CI: 0.44-0.92) with thiopurines compared to placebo[42].

Other trials have compared thiopurine to mesalamine formulation. However, superiority of a thiopurine over mesalamine was unable to be shown in multiple trials[41,44-47]. It is possible that side effects often prohibit adherence of thiopurines[46]. The meta-analysis by Doherty *et al*[42] reported a higher risk of treatment discontinuation due to side-effects in patients taking thiopurines. Long term data from a retrospective analysis demonstrated that thiopurine treatment for over 36 mo decreased surgical recurrence compared to treatment less than 36 mo or no treatment at all[48]. Thus long-term maintenance may be beneficial especially in those who can tolerate the treatment, but the side effect profile may be prohibitive especially as mesalamine agents may provide a similar decreased risk of recurrence.

Table 2 Recurrence rates with each agent discussed (range) from randomized controlled trials, *vs* placebo rates

Agent	Endoscopic recurrence		Clinical recurrence	
	Drug	Placebo	Drug	Placebo
Mesalamine	5%-51%	50%	11%-16%	19%-23%
Thiopurines	2%-22%	50%	0%-36%	10%-50%
Anti-TNF	0%-21%[1]	81%-85%[1]	0%-20%	25%-46%
Antibiotics	13%	43%	7%-24%	25%-38%
Probiotics[2]	9%-21%	15%-16%	9%-17%	6%-14%
Budesonide	52%	57%	57%[3]	70%[3]

Ranges of reported endoscopic and clinical recurrences for various therapies and the range of placebo reports from those trials. Endoscopic recurrence rates are for severe (≥ i2) recurrence unless otherwise stated. Follow-up time is 12 mo unless otherwise stated. [1]Any endoscopic recurrence; [2]Three to twelve months follow-up; [3]Combined clinical and endoscopic recurrence. TNF: Tumour necrosis factor.

Anti-TNF antibodies

Most recently anti-TNF therapeutic antibodies have been studied in the prevention of post-operative prophylaxis. Infliximab has been studied most, although there is some emerging data for adalimumab. Early non-controlled reports noted a decreased in both endoscopic and clinical recurrence of CD postoperatively[49]. Subsequent prospective controlled studies also demonstrated a benefit compared to placebo[50,51], mesalamine[28,52] and thiopurines[28]. However, there have only been two randomized trials comparing infliximab and both were compared with placebo. Regueiro *et al*[53] demonstrated that endoscopic lesions were significantly lower at one year in the infliximab group compared to controls (9.1% *vs* 84.6% respectively, P = 0.0006), but were unable to detect a significant difference in the clinical recurrence rate due to small numbers. A follow up abstract from the same group suggested that patients can have a benefit for up to two years, although they will relapse if infliximab is stopped[54]. Yoshida *et al*[55] did demonstrate a decrease in

the clinical remission rate at 12 mo with infliximab compared to placebo (100% *vs* 68% respectively, $P < 0.03$). Additionally adalimumab has demonstrated efficacy if prevention of postoperative recurrence although data remains small with a lack of controls[56-58]. Preliminary data from the POCER study was presented in abstract form demonstrated that at 6 mo 94% of high-risk patients treated with postoperative adalimumab were in endoscopic remission (i0-i1) *vs* 62% of high risk patients on a thiopurine ($P = 0.02$)[59]. However, patients on a thiopurine who had recurrence had an escalation of therapy to adalimumab. Thus at 18 mo, there was no difference between endoscopic recurrence in patients who received adalimumab immediately post operatively and those who had tailored therapy based on 6 mo endoscopy[60]. Thus early data from ongoing trials suggests that prophylaxis with anti-TNF antibodies may be highly effective compared to other treatments, although careful patient selection is likely required to identify whom to administer prophylaxis to.

Probiotics

Manipulation of the bacterial flora is an attractive mode of preventing postoperative recurrence, as specific bacteria including bacteroides, fusobacteria and *E. coli* have been found in increased amount in the neo-terminal ileum[14]. Both individual *lactobacillus* strains as well as probiotic cocktails have been studied. *Lactobacillus johnsonii* (LA1) and *Lactobacillus rhamnosus* GG (LGG), failed to show any benefit in prevention of recurrence[61-63]. The probiotic cocktail Synbiotic 2000 similarly failed to show a benefit compared to placebo in a randomized controlled trial[64]. Similarly the yeast *Saccharomyces* boulardii did not demonstrate prevention of relapse in a randomized controlled trial of patients in medical remission[65]. However, a small study with VSL#3 did note that patients receiving the probiotic had less severe endoscopic recurrence (lower Rutgeerts score) and reduced levels of pro-inflammatory cytokines[66]. Ultimately, pooled data failed to show any difference with regards to clinical (RR *vs* placebo: 1.41, 95%CI: 0.59-3.36) or endoscopic recurrence (RR *vs* placebo: 0.96, 95%CI: 0.58-1.58)[42].

Antibiotics

Nitroimidazole antibiotics including metronidazole and ornidazole have been shown to decrease the risk of clinical and severe endoscopic recurrence[42,67,68]. Further illustrating the usefulness of antibiotics was a trial where all patients received metronidazole for one month and either azathioprine or placebo for one year. The overall rate of recurrence was low which was attributed to the widespread metronidazole use, although in combination with azathioprine endoscopic recurrence was less frequent and less severe[43]. However, nitroimidazole antibiotics are difficult to tolerate and cessation rates are high, up to one third, limiting widespread clinical utility[67,68].

COSTS AND DURATION OF CHEMOPROPHYLAXIS

The goal of therapy for post-operative prophylaxis is to decrease clinical recurrence. Not all patients will develop clinical recurrence and so treating everyone one may not be cost effective or feasible. Two studies examined the cost and feasibility of treating patients with various medical therapies in the postoperative setting. Doherty *et al*[69] noted that incremental cost-effectiveness ratio (ICER), or costs per QALY gained, was $1.8M for infliximab, when compared with no prophylaxis. In their cost-effectiveness model, azathioprine was the most cost-effective medication strategy over 1 year, when compared to "no prophylaxis". Ananthakrishnan *et al*[70] similarly noted that initial therapy with infliximab is not cost effective although suggested that antibiotics (metronidazole or ornidazole) were cost effective when tolerated. Both of these studies made assumptions of a recurrence rate of around 20%-30% with no treatment. As the rate of recurrence increases, the cost-effectiveness of an anti-TNF increases as well.

USING ENDOSCOPIC SCREENING TO TAILOR INTERVENTIONS

Endoscopic recurrence precedes clinical recurrence, but not all endoscopic recurrence leads to clinical recurrence. Endoscopic changes are often noted as early as 2 mo following surgery[71]. Endoscopic recurrence rates vary widely at 1 year without any treatment ranging from 28%-93%[72]. Overall, it is estimated that 20% of patients with endoscopic recurrence will progress to clinic recurrence, although the severity of the endoscopic recurrence can predict the likelihood of clinical recurrence[8,19]. Over a four year follow-up period, 100% of patients with severe endoscopic recurrence (Rutgeerts scope of i2-i4) developed symptomatic recurrence compared to only 9% of patients with a low score (i0-i1)[73]. Thus evaluation of a patient within the first year following resection of CD can effectively stratify patients at a high risk of clinical recurrence. Patients who develop a high Rutgeerts score ($\geq$ i2) who are then treated with infliximab have decreased clinical activity compared to treatment with thiopurines or mesalamine[28]. One study demonstrated that an endoscopically-tailored therapy (with any medical therapy) has a similar time to symptomatic recurrence compared to immediate post operative prophylaxis[74]. Recently, preliminary results of the POCER study were presented, supporting the use of endoscopically tailored therapy. In this trial, all patients received three months of antibiotics and high-risk patients (smoker, penetrating disease, $\geq$ second operation) received a thiopurine (or every other week adalimumab if thiopurine intolerant). Patients were then randomized to active care with a colonoscopy at 6 mo and step up of therapy if evidence of recurrence (Rutgeerts score $\geq$ i2) or standard of care. At

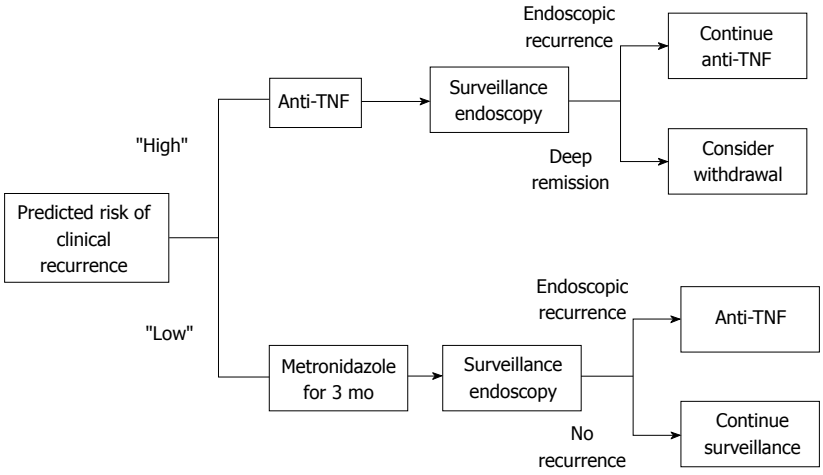

Figure 1 Suggested algorithm for deciding when to administer post-operative prophylaxis based on effectiveness of treatment. Other considerations such as cost and prior success or failure of treatment need to be individualized. Patients at high risk are those who have 2 or more of the following risk factors: smoking, penetrating disease, history of prior resection, and myenteric plexitis. Based on the available evidence, we suggest first surveillance endoscopy be done at 6 mo time for all patients. Treatment escalation at 6 mo should be considered for all patients with evidence of endoscopic recurrence (Rutgeerts ≥ i2). For select patients who achieve and maintain deep remission on therapy (Rutgeerts of i0 with normal histology), consideration can be given to de-escalation of therapy to either thiopurine alone or close monitoring.

18 mo, significantly less endoscopic recurrence was seen in the active care group *vs* the standard of care group (49% *vs* 67% respectively, $P = 0.028$)[75].

An interesting option to explore in the future is immediate prophylaxis followed by endoscopic assessment and cessation of treatment if no endoscopic recurrence or other surrogate marker for inflammations such as serum inflammatory markers or fecal calprotectin. One retrospective cohort demonstrated that patients who achieve clinical remission on infliximab and maintain maintenance therapy for one year have a 69% probability of being disease free at 1 year after infliximab discontinuation[76]. The STORI trial is a prospective cohort following a group of patients who have received > 1 year of therapy with infliximab and an immunomodulator and achieved steroid free remission who then stop infliximab. Over 50% of patients will not relapse at one year and there may be a subgroup of patients in deep remission who do even better[77]. Extrapolating this premise and applying it to patients who achieve surgical remission followed by a year of therapy is appealing and potentially cost effective. One small study has evaluated one year of infliximab for chronic refractory pouchitis following colectomy for ulcerative colitis. Notably the patients who responded completely to infliximab were able to discontinue after one year without recurrence at follow-up[78].

IMPLICATIONS FOR PRACTICE

The questions of if and when to treat in the post-operative setting, as well as the optimal therapeutic regimen, remains incompletely answered. Each patient requires an individual risk assessment as well as careful consideration to prior therapies and what led them to surgery[4]. Overall, tailored therapy is advisable as a general rule given the cost and side effect profile of the majority of treatments. However, in the highest risk patients (*i.e.*, penetrating disease, history of prior resection, current smoking), upfront infliximab use provides the greatest benefit although it is not considered cost effective[70]. As long-term data on infliximab becomes available, the balance of cost and prevention of disease may change. Additionally as data becomes available for other anti-TNFs, this model will need to be updated. Thus for the highest risk patients, the decision for upfront anti-TNF *vs* tailored therapy remains unclear and must likely be individualized. If the decision for tailored therapy is made, then endoscopic evaluation should take place at 6 mo time as suggested by the recent POCER data[60,75]. In patients who are not high risk, then an empiric course of nitroimidazole antibiotic is likely to be cost effective, if the patient can tolerate the therapy, with or without maintenance thiopurine followed by endoscopic assessment at 6 mo time[4,60,70,75]. A proposed algorithm for post-operative prevention is shown in Figure 1. While this approach is the most efficacious based on the available evidence, it is not the most cost effective and the decision for upfront anti-TNF therapy or tailored therapy in high risk patients will need to be individualized.

The optimal treatment regimen is not known and likely will depend heavily on the patient's prior treatments. The biologic naïve patient may achieve the best effect with an anti-TNF, although the cost effectiveness of long-term prevention may not be favorable. While the majority of data is with infliximab, it appears that adalimumab will be efficacious as well, necessitating further cost effective analysis. Postoperative prophylaxis in CD does decrease clinical recurrence. However the optimal timing and type of therapy remain unknown. Stratifying patients according to risk of symptomatic recurrence and tailoring therapy is the ideal and most cost effective way to treat patients, however these questions have not been fully answered.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Role of capsule endoscopy in inflammatory bowel disease

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Abstract

Videocapsule endoscopy (VCE) has revolutionized our ability to visualize the small bowel mucosa. This modality is a valuable tool for the diagnosis of obscure small bowel Crohn's disease (CD), and can also be used for monitoring of disease activity in patients with established small-bowel CD, detection of complications such as obscure bleeding and neoplasms, evaluation of response to anti-inflammatory treatment and postoperative recurrence following small bowel resection. VCE could also be an important tool in the management of patients with unclassified inflammatory bowel disease, potentially resulting in reclassification of these patients as having CD. Reports on postoperative monitoring and evaluation of patients with ileal pouch-anal anastomosis who have developed pouchitis have recenty been published. Monitoring of colonic inflammatory activity in patients with ulcerative colitis using the recently developed colonic capsule has also been reported. Capsule endoscopy is associated with an excellent safety profile. Although retention risk is increased in patients with small bowel CD, this risk can be significanty decreased by a routine utilization of a dissolvable patency capsule preceding the ingestion of the diagnostic capsule. This paper contains an overview of the current and future clinical applications of capsule endoscopy in inflammatory bowel disease.

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Key words: Small bowel videocapsule endoscopy; Crohn's disease; Pouchitis; Indeterminate inflammatory bowel disease; Ileal pouch-anal anastomosis; Patency capsule

Core tip: Videocapsule endoscopy has revolutionized our ability to visualize the small bowel mucosa. This modality is a valuable tool for the diagnosis of obscure small bowel Crohn's disease (CD), and can also be used for monitoring of disease activity, detection of complications, evaluation of therapeutic response and postoperative recurrence in established CD, evaluation of the small bowel in patients with unclassified inflammatory bowel disease and pouchitis. Monitoring of colonic inflammation in patients with ulcerative colitis has also been reported. This manuscript contains an overview of the current and future clinical applications of capsule endoscopy in inflammatory bowel disease.

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INTRODUCTION

In the past, the small bowel has largely been inaccessible to direct endoscopic examination, with only the duodenum, proximal jejunum and terminal ileum being subject to direct visualization by a conventional endoscope. This paradigm changed dramatically with the invention and introduction of small bowel videocapsule endoscopy

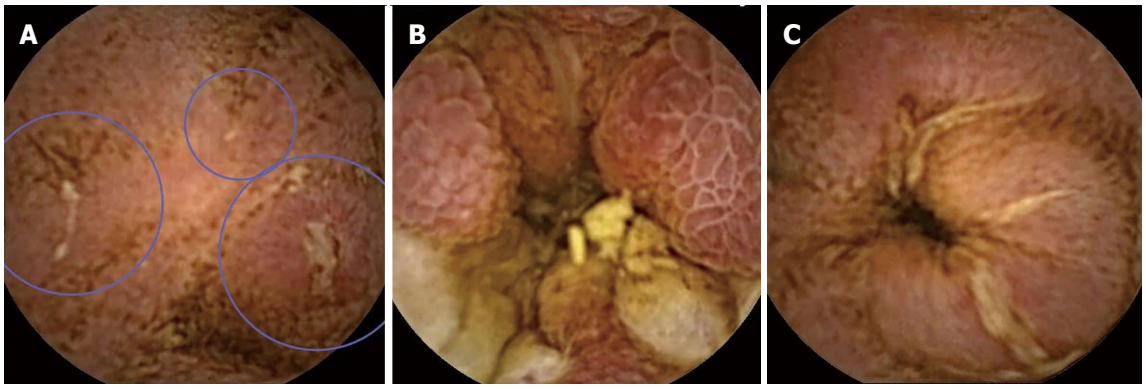

Figure 1 Videocapsule endoscopy findings. A: Small ulcers (encircled); B: Edematous mucosa; C: Ulcerated stenosis (SBII capsule, RAPID and Imaging software, Given Imaging, Yokneam, Israel).

(VCE) in 2000[1]. The first wireless capsule, manufactured by Given Imaging (Yokneam, Israel) was approved for clinical use in United States and Europe in 2001[2]. Several other manufacturers subsequently released their own versions of VCE. This technology has been extensively used for the diagnosis and monitoring of patients with inflammatory bowel disease (IBD), mostly Crohn's disease (CD). About 30% of the patients with CD have exclusive small bowel involvement[3], and their diagnosis will frequently be missed if based solely on ileocolonoscopic findings. VCE is now also considered an important technique for monitoring small bowel CD, and has also been employed in management of patients with unclassified IBD and ulcerative colitis.

The aim of the current review is to outline the diagnostic role of VCE in the diagnosis and monitoring of inflammatory bowel disease, in particular small-bowel CD.

DIAGNOSIS OF CD

Characteristic endoscopic findings

Several VCE findings are frequently associated with CD: ulcerations, erythema, mucosal edema, loss of villi, strictures and mucosal fissures (Figure 1)[4]. Unfortunately, none of these findings is specific for CD. In fact, minor small bowel lesions maybe present in up to 10% of normal subjects[5]. As VCE lacks tissue-sampling capabilities, it cannot confirm the etiology of the observed lesions. The most common mimicker of CD in the small bowel is non-steroidal anti-inflammatory medication (NSAID)-induced enteropathy that may manifest with lesions indistinguishable from those of CD. Such lesions, appearing as early as 2 wk from the onset of NSAID therapy, can be demonstrated in 70% of chronic NSAID users[6,7]. Thus, VCE should be reserved for patients with high clinical index of suspicion for CD. Patients who are candidates for VCE should be instructed to avoid NSAIDs for at least 1 mo before the examination. Similar bowel mucosal lesions may result from multitude of other pathologies, such as lymphoma, radiation enteritis, HIV with opportunistic infection, intestinal tuberculosis and Behcet's disease[5].

Diagnostic scores

The criteria for diagnosis of CD using VCE have not been well established. The most commonly used validated diagnostic index is the Lewis score[8]. This score divides the small bowel into 3 tertiles (dividing the small bowel transit time in 3) and uses an algorithm that assigns points to various findings (mucosal edema, ulcers, strictures) characteristic for CD in each of the tertiles, taking in account the severity and the reproducibility of each finding. The final score represents the number of points accumulated by the most significantly involved tertile. The Lewis score is incorporated in the software used for decoding, reading and interpretation of VCE images obtained by PillCAM (RAPID). A score < 135 is designated as normal or clinically insignificant mucosal inflammatory changes, a score between 135 and 790 indicates mild, and a score ⩾ 790 moderate to severe inflammation, respectively. An additional score known as capsule endoscopy CD activity index (CECDAI or Niv score), was recently proposed (Table 1)[9]. This score incorporates three main characteristics of CD: inflammation, extent of disease, and strictures, in both the proximal and distal segments of the small bowel. It should be noted that while these scores attempt to quantify the severity and extent of small bowel (SB) CD, the lesions are not pathognomonic and may represent other causes of bowel inflammation.

VCE was also utilized for diagnosis of SB CD in patients primarily presenting with extraintestinal manifestations of IBD. Arhtropathy is the most common extraintestinal manifestation in IBD, occurring in 6%-46% of the patients[10], and frequently manifesting even before the onset of bowel disease. Capsule endoscopy may be a valuable tool in evaluation of these patients, especially if conventional ileocolonoscopy is unappealing to the patient[11]. Spondyloarthroathy can be diagnosed in up to 30% of IBD patients[12,13]. Capsule endoscopy can demonstrate small bowel lesions consistent with CD in 33% of these patients (twice as many as conventional ileocolonoscopy[14]).

VCE vs other modalities for the diagnosis of CD

The yield of VCE for the diagnosis of CD was com-

Table 1 A comparison of 2 capsule endoscopy scoring indices for quantification of mucosal inflammation

Parameter	Lewis score[8]			CECDAI[80]	
	Number/quality	Longitudinal extent	Descriptors	Parameter	Descriptors
Villous appearance	Normal/edematous	Short segment/long segment/whole tertile	Single/patchy/diffuse	Inflammation score	None to large ulcer (> 2 cm)
Ulceration	Non/single/few/multiple	Short segment/long segment/whole tertile	< 25%, 25%-50%, > 50%	Extent of disease	No disease to diffuse (3 segments)
Stricture	Non/single/few/multiple	Ulcerated/non-ulcerated	Traversed/non-traversed	Stricture score	None to complete obstruction
Small bowel segmentation	Tertiles (strictures for the entire length of the examination)			Proximal to distal small bowel	
Score	< 135: Normal or clinically insignificant inflammation 135-790: Mild inflammation 790: Moderate to severe.			0 (normal examination)-26 (severe inflammation)	

CECDAI: Capsule endoscopy Crohn's disease activity score.

Table 2 Key studies evaluating the diagnostic yield of capsule endoscopy for Crohn's disease

Modality	Ref.	Number of patients	Diagnostic yield of VCE	Diagnostic yield of the compared modality	IY	*P* value
CTE	Eliakim *et al*[81]	35	77%	20%	47%	< 0.05
	Hara *et al*[82]	17	71%	53%	18%	NA
	Voderholzer *et al*[83]	41	61%	49% (CT enteroclysis)	12%	< 0.04
	Solem *et al*[84]	40	83%	83%	0	NS
MRE	Albert *et al*[85]	27	93%	78%	15%	NS
	Crook *et al*[22]	19	93%	71%	18%	NS
	Jensen *et al*[20]	93	100%	86%	14%	NS
Ileocolonoscopy	Hara *et al*[82]	17	71%	65%	6%	NS
	Solem *et al*[84]	40	83%	74%	9%	NS
	Leighton *et al*[86]	80	55%	25%	30%	NA

VCE: Videocapsule endoscopy; CTE: Computed tomography enterography; MRE: Magnetic resonance enterography; IY: Incremental yield; NA: Not available; NS: Non significant.

pared to that of cross-sectional imaging modalities such as small bowel follow-through (SBFT), computer tomography enterography (CTE) and magnetic resonance enterography (MRE) in multiple studies (Table 2). Patients with suspected small bowel stenosis were excluded from VCE evaluation in these studies. The superiority of VCE over small bowel follow-through and enteroclysis has been repeatedly demonstrated in multiple studies[15-18].

A recent meta-analysis demonstrated an incremental diagnostic yield (IY) of VCE in comparison to CTE in both suspected and established CD patients (IY, 47%; 95%CI: 31%-63%, $P < 0.00001$; and 32%; 95%CI: 16%-47%, $P < 0.0001$), respectively[19]. A prospective trial evaluated the diagnostic accuracy of VCE, MRE and CTE in 93 patients with suspected CD as compared to ileocolonoscopy. The sensitivity and specificity for diagnosis of CD of the terminal ileum was 100% and 91% by CE, 81% and 86% by MRE, and 76% and 85% by CTE, respectively. There was statistical difference in sensitivity compared with CTE, but only a trend in comparison with MRE. Specificity was not significantly different between the modalities. Proximal small bowel CD was detected in 18 patients by using CE, compared with 2 and 6 patients using MRE or CTE, respectively ($P < 0.05$)[20]. In earlier studies, VCE and MRE were reported to have comparable accuracy. Overall, VCE is more accurate in diagnosing subtle small bowel lesions and MRE in diagnosing intramural inflammation, stricturing complications and extra-intestinal manifestations[19,21,22]. The superior sensitivity of VCE clinical for proximal small bowel disease is a potentially important diagnostic advantage, as proximal small bowel disease has recently been demonstrated to be a significant negative prognostic factor[23].

Importantly, data acquired by different endoscopic and imaging modalities can be combined to improve the diagnostic accuracy, utilizing the specific advantages and strengths of each modality.

VCE in established CD

VCE is a potentially important but currently underutilized tool for monitoring of SB CD. In the latter years, the leading treatment paradigm in IBD has shifted form merely controlling symptoms to reversing the underlying inflammation, as expressed by objective surrogate markers such as laboratory inflammatory markers and endoscopic evidence of mucosal healing[24]. Capsule endoscopy provides meaningful information on the inflammatory burden in the small bowel mucosa, similarly to the role of conventional ileocolonoscopy for the colon and the terminal ileum. Bowel stenosis should be ruled

Table 3 Key studies describing the role of videocapsule endoscopy in established Crohn's disease

Indication	Ref.	*n*	Inclusion criteria	Diagnostic criteria	Results
Mucosal healing	Efthimyou *et al*[28]	40	Patients with active CD (CDAI > 150) who responded to anti-inflammatory treatment, VCE was performed before and after treatment	Number of aphthous ulcers/large ulcers/length of involved segment	Only number of large ulcers correlated with response (8.3 ± 1.4 and 5/0.8, 95%CI: 0.8-5.9, $P < 0.01$)
Postoperative recurrence	Bourreille *et al*[31]	31	CD with ileocolonic anastomosis	Rutgeerts score ⩾ 1	VCE-21/31 (68%), IC-19/31 (61%)
	Pons Beltrán *et al*[32]	24	CD with ileocolonic anastomosis	Rutgeerts score ⩾ 2	VCE-14/22 (55%), IC-6/24 (25%)
Unexplained symptoms	Dubcenco *et al*[34]	28	Active CD patients	⩾ 3 ulcers	VCE-23 (82%), IC-14 50%, barium radiography-9 (32%)
	Dussault *et al*[35]	25	Active CD patients with unexplained symptoms	Severity graded by number and appearance of ulcers and presence of stenosis	Active SB inflammation: 11/25 (44%)

In 6 patients treated with immunomodulators, biologics or corticosteroids, a significant improvement was demonstrated in all 3 parameters. CD: Crohn's disease; VCE: Vidoecapsule endoscopy; IC: Ileocolonoscopy; SB: Small bowel.

out before VCE is performed in established CD due to the increased risk of capsule retention (about 5%). Routine use of patency capsule diminishes the risk of retention to almost negligible (see below).

VCE could be particularly useful in the following clinical scenarios in known CD (Table 3): (1) Monitoring of mucosal healing; (2) Detecting postsurgical recurrence; and (3) Discrepancy between clinical and laboratory data and endoscopic findings.

Mucosal healing: Mucosal healing, defined as absence of visible endoscopic inflammation, has emerged as a very important marker of long-term clinical efficacy associated with decreased risk of long-term complications in both ulcerative colitis (UC) and CD[24-27]. Conventional ileocolonoscopy is the current gold-standard modality for assessment of mucosal healing. A small prospective study had evaluated monitoring of mucosal healing with VCE performed before and after treatment for acute CD flare-up[28]. Forty patients with CD flares were included in the study and all have responded to treatment within 4-8 wk of treatment. Three parameters (number of large ulcers, number of aphthous ulcers and percentage of time with lesions visible) were examined. Of these only the first one improved significantly. In a subgroup of patients treated with corticosteroids combined with immunomodulators or biologics, a significant improvement in all three parameters was demonstrated. The most important limitations of this study were a significant heterogeneity in the instituted treatment, with majority of patients treated with mesalamine or corticosteroids, along with absence of a validated scoring system for mucosal inflammation. The data from our center demonstrated a significant reduction in the Lewis score in 4 patients with spondyloarthropathy and newly diagnosed SB CD after 6 mo of treatment with Adalimumab[14]. Importantly, no diagnostic score, including the commonly used Lewis score, has been validated for evaluation of mucosal healing in SB CD.

Postoperative CD recurrence: Recurrence of SB CD in the neo-terminal ileum following surgical resection can be demonstrated in 73%-93% of the patients within 1 year of ileocolonic resection[29,30]. SB lesions associated with postoperative recurrence are frequently quantified using the Rutgeerts score[29]. The accuracy of VCE in detection of postoperative recurrence was evaluated in 31 patients[31]. Recurrence occurred in 21 patients (68%) and was detected by ileocolonoscopy in 19 patients. Sensitivity of VCE using the Rutgeerts score was 62%-76% and specificity was 90%-100%. The severity of lesions as assessed by both methods correlated significantly ($P < 0.05$). In an additional study, 24 patients with CD, neo-terminal ileum recurrence defined as Rutgeerts score > 2 was demonstrated by ileocolonoscopy in 25% and capsule endoscopy in 62% (VCE was performed in 22/24 patients due to failure to excrete the patency capsule in 2 patients). Capsule endoscopy detected proximal SB lesions inaccessible by ileocolonoscopy in 13 patients[32]. VCE is an attractive monitoring modality for postoperative patients, providing a non-invasive and accurate visualization of the entire small bowel including the neo-terminal ileum.

Unexplained symptoms: Many symptoms of CD, such as diarrhea, abdominal pain, bloating, can be attributed to a multitude of etiologies other than active inflammation [underlying irritable bowel syndrome (IBS), bacterial overgrowth, bile salt diarrhea *etc*.]. Clear identification of inflammatory etiology is of crucial importance and may lead to significant changes in the treatment, such as initiation or escalation of anti-inflammatory treatment. Negative VCE results are also of clinical importance, as this would lead to diagnosis and initiation of treatment for a concomitant condition such as IBS, and prevent further unnecessary and expensive escalation of an anti-inflammatory regimen. Clinical indices and laboratory inflammatory markers may indicate ongoing inflammation, but lack sensitivity. In a study including 140 patients with CD, the Spearman's rank correlation of simple endoscopic index with fecal calprotectin, CRP, blood leukocyte count and CDAI was 0.75, 0.53, 0.42 and 0.38, respectively[33]. Although ileocolonoscopy is a gold standard test for identification of active inflammation, it would potentially miss lesions located proximally to the ileocecal valve.

Table 4 Key studies describing the role of videocapsule endoscopy in unclassified Inflammatory bowel disease pouchitis and ulcerative colitis

	Ref.	Indication	*n*	Definition of CD	Results *n* (%)
IBD-U	Mehdizadeh *et al*[41]	IBDU	6	> 3 ulcerations-diagnostic of CD, 1-2 ulcerations-suggestive of CD	1 (17) SB findings
	Maonoury *et al*[42]	IBDU	30	> 3 ulcerations-diagnostic of CD, 1-2 ulcerations-suggestive of CD	5 (17), CD
	Cohen *et al*[43]	IBDU	2	NA	1 (50), CD
s/p IPAA	Mow *et al*[40]	Isolated colitis	6	> 3 ulcerations-diagnostic of CD, 1-2 ulcerations-suggestive of CD	3 (50)-definite CD, 2 (20), possible CD
	Mehdizadeh *et al*[41]	Persistent symptoms after IPAA	21	> 3 ulcerations-diagnostic of CD, 1-2 ulcerations-suggestive of CD	7 (33%) SB findings
	Calabrese *et al*[47]	Chronic pouchitis after IPAA	15	NA	Gastric or SB lesions, 15 (100)
Ulcerative colitis	Mow *et al*[40]	Isolated colitis	12	> 3 ulcerations-diagnostic of CD, 1-2 ulcerations-suggestive of CD	3 (25)-definite CD, 3 (25), possible CD
	Mehdizadeh *et al*[41]	Treatment-resistant UC	22	> 3 ulcerations-diagnostic of CD, 1-2 ulcerations-suggestive of CD	2 (9), SB findings
	Cohen *et al*[43]	UC	5	NA	4 (80), CD
	Higurashi *et al*[44]	UC	23	Lewis score	13 (56.5), small bowel lesions 9 (39), Lewis score > 135

IBDU: Unclassified inflammatory bowel disease; IPAA: Ileoanal pouch anastomosis; CD: Crohn's disease; UC: Ulcerative colitis; SB: Small bowel.

Dubcenco *et al*[34] have prospectively evaluated 28 symptomatic Crohn's patients with ileocolonoscopy, barium radiography and capsule endoscopy. Active disease was identified by VCE, ileocolonoscopy and barium radiography in 82%, 49% and 32% of patients, respectively. In a study by Dussault *et al*[35], in 25 out of the included symptomatic CD patients, VCE was indicated for a discrepancy between clinical symptoms and diagnostic findings. Abnormal SB findings were diagnosed in 44% of the patients, and in 45% of these patients the treatment was escalated following the performance of VCE.

VCE can also be used for monitoring of ileal recurrence in CD patients following bowel resection and ileocolonic anastomosis. In one study, VCE detected CD recurrence in 15 (62%) patients, whereas ileocolonoscopy detected inflammatory lesions in the neo-terminal ileum in only 6 (25%) patients[32]. VCE was also evaluated for a potential role in the assessment of mucosal healing after drug therapy in CD[28].

Therapeutic yield of VCE in established CD

VCE frequently produces clinically significant data that can lead to a change in a therapeutic management. In a retrospective series of 71 CD patients, medical treatment was changed in 38 (53%) of the patients within 3 mo of VCE performance[35]. In an additional series that included 86 patients with established CD, 61.6% had a change in medication in the 3 mo after the CE, with 39.5% initiating a new anti-inflammatory medication[36].

VCE in unclassified IBD and UC

Colonic inflammatory bowel disease cannot be classified as CD or UC using current colonoscopic and pathologic criteria in 10%-15% of the patients[37]. At least 30% of these patients with unclassified IBD (IBDU) will be reclassified as CD during the course of their illness[38], usually after identification of small bowel lesions. Correct classification of the patients is especially important when deciding on surgical intervention, as rates of chronic pouchitis, fistula formation and pouch failure after ileal pouch-anal anastomosis (IPAA) are significantly higher in patients with CD[39].

Several small studies have evaluated the utility of VCE for reclassification of IBDU patients. Mow *et al*[40] have described 22 patients with either isolated colitis or chronic symptoms following IPAA (n = 18) who were evaluated with VCE. All patients had prior unremarkable small bowel radiography. Multiple ulcerations (3 and above) considered diagnostic for CD were identified in 68 of 18 patients. Mehdizadeh *et al*[41] described 120 patients with a history of UC or IBDU who underwent VCE. Findings consistent with SB CD were demonstrated in 15.8% of the patients. Eighteen/19 patients with CD diagnosed by VCE have previously underwent SBFT, with positive findings in only 1 patient. Another series included 30 patients with IBDU, in whom CD (defined as 3 or more SB ulcers) was identified in 5. Interestingly, in 6/25 VCE-negative patients CD was diagnosed on a subsequent ileocolonoscopy with biopsies[42]. In a series of pediatric patients, 5/7 patients initially diagnosed as UC or IBDU were reclassified as having CD as a result of VCE findings[43] (Table 4).

Higurashi *et al*[44] evaluated small bowel inflammation in patients with established UC. Of the 23 UC patients, 13 (57%) showed small-bowel lesions, and 8 (35%) had erosions, as opposed to 2/23 (7%) and 1/23 (4%) in the control group. In 9/23 patients with UC, the Lewis score of inflammation was consistent with mild to moderate small bowel inflammation (between 135 and 790). The clinical and pathological significance of these lesions is unclear (repeated biopsies were performed in only 2 patients, but these results are of great interest and em-

phasize the possible risk of misdiagnosis in many IBD patients.

VCE in evaluation of pouchitis in patients after IPAA

IPAA provides a continence-preserving surgical option in patients with UC unresponsive or unwilling to continue anti-inflammatory therapy, or those who have developed complications (such as colonic stenosis, colonic dysplasia *etc.*) that require total colectomy. The procedure is technically demanding and is associated with a significant incidence of postoperative complications, the most common being chronic and acute pouchitis and "*de novo*" CD[45]. Symptoms and endoscopic lesions consistent with chronic pouchitis are reported in 10%-59% in patients with UC, and even more frequently in patients with CD[46]. It is commonly argued that at least a subgroup of these patients actually represent a previously undiagnosed CD. In a series of 15 UC patients with chronic pouchitis, diffuse lesions involving the stomach or different segments of the small bowel were demonstrated in all patients[47]. Similar lesions were demonstrated in 27% of the control patients. Unfortunately, histological evaluation (showing non-specific inflammation) was available for only 2 patients with gastric involvement. The clinical significance of these lesions is unclear (Table 4).

The role of VCE in preoperative evaluation of UC/IBDU patients was examined in one retrospective series. The study evaluated the incidence of acute pouchitis, chronic pouchitis and *de novo* CD within 12 mo of the surgery in patients with and without pathological findings on a preoperative VCE. No significant difference was demonstrated for any of the outcomes[48]. However, an important limitation of this study was the definition of "positive" VCE as any ulceration or lesion, possibly leading to a high false positive rate. In addition, a significant selection bias stemming from the retrospective design of the study (patients with a high preoperative probability of CD were not likely to undergo IPAA) interferes with the interpretation of the results. In our opinion, preoperative evaluation of IPAA candidates with VCE merits further evaluation in prospective studies.

Anemia is another frequent complication of IPAA surgery, occurring in about 17% of UC patients[49]. Possible etiologies may include newly discovered CD, arteriovenous malformations, celiac disease and others. In a series of post-IPAA patients with chronic anemia, VCE detected the cause of anemia in 29.4%. Sixty percent of the patients were diagnosed as having a new-onset CD[50].

COLONIC CAPSULE ENDOSCOPY IN UC

A colonic capsule [PillCam colonic capsule (PCCE) Given Imaging, Yokneam, Israel] has been available for colorectal cancer screening for several years. This device includes 2 cameras which records 2 different sets of images. The colonic capsule was compared with colonoscopy with promising results, with the second-generation capsule reaching sensitivity of 88% for detection of polyps > 6 mm in comparison to colonoscopy[51,52].

PCCE was evaluated for diagnosis and monitoring of UC. In the study by Ye *et al*[53], 25 patients were evaluated for presence and severity (Mayo Score) of UC by PCCE and conventional colonoscopy. A significant correlation in the severity (k = 0.751, $P < 0.001$) and extent (k = 0.522, $P < 0.001$) of UC between the PCCE and conventional colonoscopy was demonstrated. Similar findings were reported by Hosoe *et al*[54]. However, PCCE is not suitable for monitoring of dysplasia and cancer surveillance in UC patients due to its lack of tissue sampling ability.

Contraindications and risks

The main complication of CE is capsule retention, defined as a failure to excrete the capsule for 2 wk or more, requiring directed medical, endoscopic or surgical intervention[55]. CE is contraindicated in patients with known bowel strictures or swallowing disorders, and history of bowel obstruction. Recent abdominal surgery is a relative contraindication[56]. In patients with obstructive symptoms or one of the aforementioned risk factors, cross-sectional imaging should be performed before VCE; however, absence of strictures on cross-sectional imaging does not preclude capsule retention[57]. The rate of capsule retention depends on the indication for performance of VCE[58]: 0% in healthy controls[59], 1.4% in obscure gastrointestinal bleeding[60-62], 1.48% in suspected CD[63-65], 5%-13% in known CD[40,66] and 21% in suspected small bowel obstruction[67]. Slow transit of the capsule, with delayed excretion of the capsule is very common, seen in up to 20% of the cases[56]. A retained capsule is usually asymptomatic[68], but may be associated with symptoms of partial or complete bowel obstruction. Only 6 cases of bowel perforation were reported[56,69]. Usually, the retained capsule can be extracted with surgery or enteroscopy. If the cause is an inflammatory stricture, corticosteroids have been useful in some cases. No consensus on the timing of intervention exists, and it is unclear how long one should wait before intervention in asymptomatic patients.

Patency capsule

The patency capsule has the same shape and dimensions as the real videocapsule. It is constructed of cellophane with wax plugs at either end and it contains lactose mixed with 10% barium to make it radiopaque. The wax plugs have holes that allow succus entericus to dissolve the lactose, resulting in capsule disintegration[70]. The dissolution of the patency capsule (Agile, Given Imaging) starts to occur after 30 h. The patency capsule can be detected by radiography or a portable radiofrequency scanner. When the patency capsule is successfully excreted or not detectable on radiography in the small bowel at 30 h post ingestion, it is usually safe to perform the diagnostic VCE. If the patency capsule location is uncertain, it is possible to localize it with the assistance of contrast or air enhanced fluorography or CT[71]. The rate of excretion of the pa-

Table 5 Potential future technological developments in capsule endoscopy relevant to inflammatory bowel disease

Manually manipulated capsule[77]
Electrically propelled capsule[78]
Tissue sampling capabilities (brushing, cytology, fluid aspiration)[87]
Therapeutic capabilities (cautery)[87]
Drug delivery

tency capsule varies from 45%-88%[58,72-75], depending upon patient selection. In a series of 77 CD patients who underwent a patency capsule examination before proceeding to diagnostic VCE, the patency capsule was not excreted within 30 h in 7.8% of the patients[35]. The main complication of patency capsule is mild abdominal pain, occurring in about 20% of the patients. Clinically evident intestinal obstruction requiring surgical intervention was reported in very few cases[58]. This phenomenon may be explained by the lodging of the capsule in sites of obstruction not easily assessable by intestinal fluids necessary for the dissolution of the lactose in the patency capsule[76]. The rate of uneventful completion of the VCE examination after successful excretion of the patency capsule approximates 100%, even though excretion times may vary between patients[58]. In cases of unsuccessful patency capsule procedure, the small bowel should be investigated by alternative diagnostic modalities such as cross sectional imaging (MR-E).

CONCLUSION

VCE possesses several important diagnostic advantages for IBD patients, mainly excellent visualization of the entire small bowel mucosa and excellent tolerability. The main challenge for further implementation of VCE in monitoring of IBD patients is an establishment of a validated quantitative score for assessment of mucosal healing and postoperative recurrence, that would allow routine utilization of this modality in both clinical practice and clinical trials. This could be especially important in CD, where outcomes in clinical trials are frequently assessed using surrogate markers (clinical scores, inflammatory markers) and evaluation of the mucosal healing limited to the colon and terminal ileum, that frequently does not reflect the inflammatory burden of the small bowel.

Small bowel lesions are frequently diagnosed in patients initially diagnosed with UC or after IPAA. The true clinical significance of these lesions, and whether they actually represent undiagnosed cases of CD is an important question that merits further clinical and translational studies.

Another important pitfall limiting the use of VCE for CD monitoring is the clinician's reluctance to perform VCE in these patients due to an exaggerated concern of retention. However, routine utilization of a patency capsule improves the safety of this procedure significantly, rendering the actual risk of retention extremely low. However, patency capsule frequently results in additional costs.

Further technological enhancements in the future may potentially lead to a further expansion of the indications for capsule endoscopy in IBD (Table 5). These improvements may include a development of an externally operated capsule, that has already been attempted[77,78]. An additional significant limitation of the capsule endoscopy is a lack of sampling ability, diminishing its usefulness for monitoring of neoplasms and colonic or small bowel dysplasia. In the future, additional technological features that are under development including tissue diagnosis capabilities, fluid aspiration, drug delivery and therapeutic (coagulation) capabilities may further increase the clinical utility of this modality[79].

VCE is a very important tool for diagnosis of CD, and also has a potentially significant role in the therapeutic monitoring of these patients. Capsule endoscopy may also provide important clinical information for patients with IBDU, UC and pouchitis, with an excellent tolerability and safety profile. Indications for VCE in IBD are likely to increase in the future with further technological and clinical developments.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Intestinal barrier in inflammatory bowel disease

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Abstract

A complex mucosal barrier protects as the first line of defense the surface of the healthy intestinal tract from adhesion and invasion by luminal microorganisms. In this review, we provide an overview about the major components of this protective system as for example an intact epithelium, the synthesis of various antimicrobial peptides (AMPs) and the formation of the mucus layer. We highlight the crucial importance of their correct functioning for the maintenance of a proper intestinal function and the prevention of dysbiosis and disease. Barrier disturbances including a defective production of AMPs, alterations in thickness or composition of the intestinal mucus layer, alterations of pattern-recognition receptors, defects in the process of autophagy as well as unresolved endoplasmic reticulum stress result in an inadequate host protection and are thought to play a crucial role in the pathogenesis of the inflammatory bowel diseases Crohn's disease and ulcerative colitis.

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Key words: Intestinal barrier; Antimicrobial peptide; Mucus layer; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Goblet cell; Paneth cell

Core tip: An efficient intestinal mucosal barrier is critical for protection against invading microorganisms, therefore impairments in this system have serious adverse effects on health. In patients suffering from the inflammatory bowel diseases Crohn's disease and ulcerative colitis, the intestinal barrier function is compromised at different levels including, amongst others, a defective production of antimicrobial peptides, alterations of the mucus layer and defects in the process of autophagy. In this article, we outline important components of the healthy intestinal mucosal barrier and review their disturbances in inflammatory bowel disease.

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INTRODUCTION

Besides its most obvious function - digestion and nutrient absorption - the intestinal tract is challenged with another complex and difficult task. As the gut lumen harbors an enormous number of microorganisms, the host has to provide a peaceful coexistence with this diverse microbial community. The successful handling of this challenge includes on the one hand prevention of an inordinate immune response against commensals and on the other hand a proper detection and elimination of pathogenic microorganisms[1-4]. The intestinal colonization increases from proximal to distal and reaches the enormous number of 10^{11}-10^{12} organisms per gram of luminal content in the colon[5,6]. To prevent a harmful adhesion and invasion of microorganisms, the intestinal mucosa is equipped with diverse specific and unspecific protective mechanisms that collectively build a complex

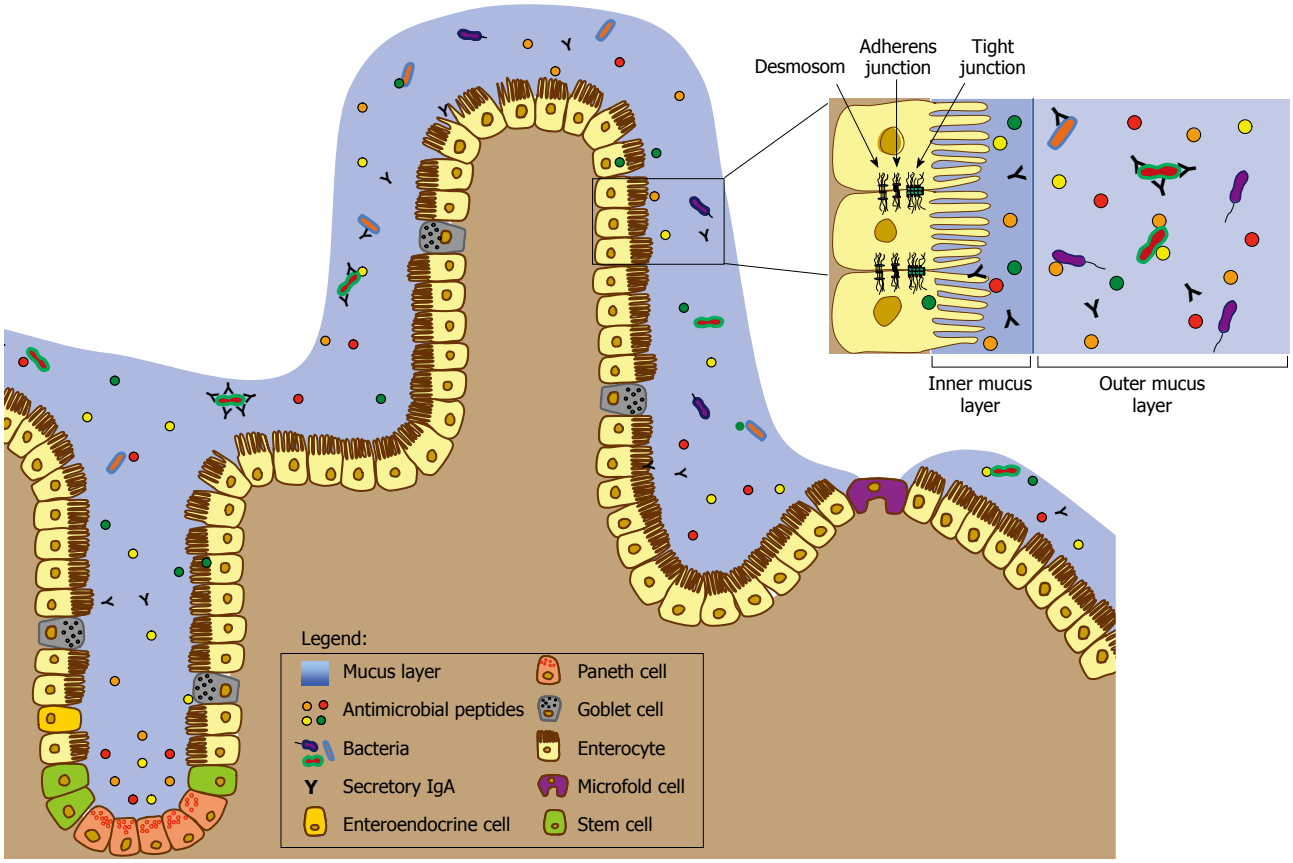

Figure 1 Defense mechanisms of the healthy intestinal mucosal barrier.

and effective mucosal barrier[7-9] (Figure 1). Some important parts of this barrier concerning the innate immunity will be discussed in this article.

COMPONENTS OF THE INTESTINAL MUCOSAL BARRIER

The epithelium

The intestinal epithelium lines the luminal surface of the gut as a single layer of closely adhering cells and forms an efficient physical boundary between luminal contents and the bodys interior[2,4,10]. The appearance of the small intestinal and the large intestinal epithelium differs in that the surface area of the small intestinal epithelium is highly increased by the formation of a large number of protrusions (folds, villi, microvilli) to accomplish the task of nutrient absorption. Situated between the villi there are glandular invaginations that are called crypts of Lieberkühn. In contrast, the epithelium of the colon has a significantly less enlarged and flat surface since it lacks the villi[2,11].

The most abundant cell type of the intestinal epithelium is the absorptive enterocyte[8,12]. Apart from enterocytes there are three different cell types with specific secretory functions, namely goblet cells, enteroendocrine cells and Paneth cells. Goblet cells are specialized in the secretion of mucus constituents and enteroendocrine cells secrete peptide hormones that are involved in cellular trophism, tissue repair, angiogenesis, enterocyte differentiation and polarization along the crypt-villus axis[3,11]. The third secretory cell-type of the intestinal epithelium, the Paneth cell, is named after the Austrian physiologist Joseph Paneth. Paneth cells contain a large number of secretory granules filled with large amounts of antimicrobial active substances like lysozyme, secretory group Ⅱ A phospholipase A2, α-defensins and the C-type lectin regenerating islet-derived protein 3-alpha (also known as HIP/PAP, hepatointestinal pancreatic protein), of which the α-defensins are by far most abundant. Paneth cells are long-lived cells and normally are confined to the small intestine where they are localized at the bottom of the crypts of Lieberkühn[1,13,14]. Furthermore, the intestinal epithelium contains microfold-cells that are part of the follicle-associated epithelium and responsible for the transport of luminal bacteria and antigens to subjacent immune cells[4,7].

The intestinal epithelium provides a selective permeable barrier as it allows the passage of water, electrolytes and dietary nutrients but prevents a detrimental invasion of foreign antigens, microorganisms and their toxins[15]. In order to fulfill this challenge, intestinal epithelial cells are closely interconnected by different protein complexes comprising tight junctions, adherens junctions and desmosomes. These cell-cell connections are required to stabilize the mechanical cohesion of the cells, to define the border between the apical and the basolateral mem-

brane regions and are also essential for the regulation of the paracellular permeability. In all three types of adhesion complexes, the cell-cell contact is mediated by an interaction between the extracellular region of specific transmembrane proteins, additionally the intracellular region of these proteins is connected *via* adaptor proteins to the cytoskeleton[11,15]. Four types of integral membrane proteins, namely occludin, the claudins, tricellulin and junctional adhesion molecules, are the main protein components of tight junctions[16].

Epithelial homeostasis for an effective intestinal barrier is maintained by the balance of cell proliferation and epithelial apoptosis. Important regulators of this homeostasis are the transforming growth factor (TGF)-α, stimulating proliferation, and TGF-β inhibiting cell growth *via* signalling pathways. Also an essential factor for the renewing of the intestinal epithelium is Wnt[17]. The canonical Wnt/β-catenin pathway regulates epithelial proliferation and the non-canonical Wnt5a pathway is essential for the formation of new crypts[18]. Fevr *et al*[19] could show that in the Wnt/β-catenin signalling a lack of β-catenin resulted in a differentiation of stem cells leading to the disturbance of the intestinal homeostasis.

With regard to the recognition of microbes and the initiation of appropriate immune responses of special importance are so called pattern-recognition receptors (PRRs) that are expressed by different cell types within the gastrointestinal tract including epithelial cells and immune cells. The two most investigated families of PRRs are the intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and the membrane bound toll-like receptors (TLRs). In general, PRRs detect microorganisms by conserved structural motifs that are characteristic for them, for example lipopolysaccharide, peptidoglycan, bacterial DNA and flagellin. Upon ligand binding, a signalling cascade is initiated that finally leads to the activation of signalling molecules like nuclear factor κB and the expression of proinflammatory cytokines, chemokines or antimicrobial peptides[4,8,20].

Antimicrobial peptides

Antimicrobial peptides (AMPs) are an integral part of the innate immunity. Their expression has been highly conserved during evolution as plants, insects, bacteria and vertebrates all make use of a variety of AMPs to protect themselves against microorganisms[21,22]. Generally, classical AMPs are small cationic peptides with an amphipathic structure[23-25]. The two best characterized families of AMPs in mammals are the defensins and the cathelicidins[25].

Characteristic features of defensins are their low molecular weight, their cationicity and a specific fold rich in β-sheet structures[26,27]. In humans, two defensin subfamilies, α- and β-defensins, are classified according to characteristic differences in their intramolecular disulfide bonding pattern[28]. The members of both subfamilies are synthesized as pre-pro-peptide that need proteolytic processing to become the mature biologically active peptides exhibiting a broad spectrum of antimicrobial activity[27,29].

Of the six so far known α-defensins, two are of particular importance in the small intestine. They are named human defensin 5 and human defensin 6 (HD5 and HD6) and in the healthy intestinal tract they are synthesized in high amounts by Paneth cells in the small intestine[30-32]. As Paneth cells are normally restricted to the small intestine, expression of Paneth cell α-defensins is absent in the healthy colon. However, in the case of inflammatory bowel disease (IBD), development of metaplastic Paneth cells and subsequent α-defensin expression in the large intestine may occur[26,33]. The four remaining members of the α-defensin family are called human neutrophil peptide 1-4 according to their principal site of expression, namely neutrophilic granulocytes[34]. There they are stored primarily as mature peptides in azurophilic granules but also in smaller amounts in their proform in specific granules[29,35]. β-defensins are mainly of epithelial origin and have been identified in various epithelia throughout the human body including the intestinal tract[36-39], the respiratory tract[40,41], and the skin[42,43]. Interestingly, environmental impacts seem to have an important influence on the functional activity of the human β-defensin 1 (HBD-1). The antimicrobial activity of this β-defensin depends to a large extent on its redox-status as reduction of disulfide bridges strongly enhanced its antimicrobial effect against the facultative pathogenic yeast *Candida albicans* as well as against anaerobic Gram-positive *Bifidobacteria* and *Lactobacilli*[44].

Some defensins are produced constitutively and provide a continuous host-defense as the β-defensin family member HBD-1. Alternatively, their expression may be inducible by proinflammatory cytokines or in the presence of microorganisms and their products. This expression pattern is shown for example the β-defensins HBD-2 and HBD-3[21,45].

The only known human cathelicidin is named LL-37 and has a broad spectrum of antimicrobial activity. The peptide is synthesized as a proform (called hCAP-18, human 18 kDa cationic antimicrobial protein) from which it is released by proteolytic processing[25,46,47]. However, studies on sweat and seminal plasma showed that a differential cleavage of hCAP-18/LL-37 is also possible, giving rise to alternative peptides with antimicrobial activity[48-50].

Apart from these typical AMPs, antimicrobial properties have been attributed to several proteins with other classical functions. For example, microbicidal activities have been described for members of the families of histones and ribosomal proteins as well as for ubiquitin[51-54].

Intestinal mucus layer

Another form of protection against invading microbes as well as other harmful insults from the intestinal lumen, including mechanical injuries or destructive enzymes, is provided by the intestinal mucus layer that coats the mucosa as a sticky gel. Apart from its protective function, mucus also has a lubricating capacity and is important to

keep the mucosal surface hydrated[55,56]. It is composed of proteins, carbohydrates and lipids and also contains a very high degree of water[57,58].

Essential for the formation of an intact and stable mucus layer are large glycoproteins belonging to the family of the secretory gel-forming mucins[59,60]. The mucin MUC2 is the predominant member of this family in the intestinal tract where it is strongly secreted by goblet cells of the small and large intestine[60,61]. By forming net-like multimeric complexes, MUC2 acts as the principal structural unit of the mucus layer. Colonic mucins carry a negative charge since their carbohydrates are substituted with numerous sulfate and sialic acid residues[62,63]. These residues offer the mucins extra protection against bacterial attack and enzymatic degradation[59,64-66].

In the large intestine, the mucus layer is divided into two distinct sectors with individual properties. The inner layer is densely packed, firmly attached to the epithelium and only removable by mechanical scraping, whereas the outer layer is not as tightly bound and can easily be aspirated with a micropipette. Interestingly, in the healthy colon the inner layer is sterile and efficiently prevents a direct contact of the epithelium with the luminal microbial community, whereas the outer layer is colonized by commensal bacteria. As studies on MUC2$^{-/-}$ mice demonstrated, formation of an efficient mucus layer is not feasible in the absence of MUC2. In a study by Van der Sluis *et al*[67], MUC2$^{-/-}$ mice developed spontaneous colitis and were more susceptible to dextran sulfate sodium (DSS) induced colitis. Moreover, Johansson *et al*[68] detected in MUC2$^{-/-}$ mice bacteria in direct contact with the epithelium, deep down in the crypts and sometimes even inside epithelial cells, whereas in wild type mice the inner mucus layer was sterile and provided an efficient spatial separation of microbes and the epithelium.

Apart from MUC2, Fc-gamma binding protein (Fcgbp) has been identified as a mucus constituent with apparent structure-providing properties. Originally, the protein was named after its capability to bind to the Fc-part of IgG antibodies. Fcgbp-expression has been detected in the mucus granules of goblet cells and the protein has been identified in large intestinal mucus. There, it is covalently attached to MUC2 and is therefore supposed to act as a MUC2-cross linker that stabilizes the MUC2 network[69-71]. Fcgbp in turn, has been identified as the disulfide linked partner of another protein-constituent of the mucus layer, the trefoil factor TFF3. It is mainly expressed in intestinal goblet cells and has been linked to mucosal protection and repair processes[72].

Moreover, the amphiphilic phospholipid phosphatidylcholine (PC) has been identified as an important mucus constituent. The interactions between PC and mucins are most likely electrostatically owing to attraction between the negatively charged mucins and the positively charged molecule regions of PC. These interactions are thought to generate a spatial arrangement where the fatty acid chains of PC are oriented to the intestinal lumen generating a protective hydrophobic surface on top of the mucus layer[73,74].

However, since the mucus layer exhibits also antimicrobial properties, it acts not only as a physical barrier. The additional function of intestinal mucus as a chemical barrier was first indicated by a study of Meyer-Hoffert *et al*[75] in which the antimicrobial properties of the murine small intestine were analyzed and a strong antimicrobial activity of mucus extracts against commensal and pathogenic bacteria was demonstrated. Moreover, various AMPs were identified in mucus extracts and, as the peptide spectrum resembled the spectrum of AMPs found in extracts from the whole small intestine as well as isolated crypts, it was assumed that epithelial AMPs are retained and thereby enriched in the murine small intestinal mucus layer. In another study, the antibacterial lectin RegIIIγ has been shown to be essential for the maintenance of a sterile zone of approximately 50 μm thickness above the murine small intestinal surface[76]. This critical contribution of an antibacterial molecule to an effective spatial segregation of luminal bacteria and the small intestinal surface also points to an antibacterial capacity of the murine small intestinal mucus layer. To enlarge the available knowledge of mucus as an antimicrobial barrier, we recently performed detailed investigations of human large intestinal mucus concerning its antimicrobial properties and revealed a high antimicrobial activity of mucus protein extracts against Gram-positive and Gram-negative bacteria as well as against the yeast *Candida albicans*. Several peptides and proteins with reported antimicrobial functions including defensins, the cathelicidin LL-37, ubiquitin and members of the family of histones could be identified in rectal mucus extracts[77]. Interestingly, several of these peptides were already identified in non-inflamed human colonic tissue in two previous studies[78,79], supporting the hypothesis that epithelial AMPs are retained in the mucus layer. Moreover, we demonstrated a binding of AMPs to mucus and mucins and also showed that this binding does not abolish the peptides functional activity[77]. As large intestinal mucins are negatively and cationic AMPs positively charged, binding of AMPs to intestinal mucus is most likely due to electrostatic interactions between these oppositely charged molecules[80,81].

Besides antimicrobial peptides, mucus also contains secretory IgA that is translocated across the epithelium in large amounts and helps to prevent an invasion of luminal microorganisms[55,82].

INTESTINAL BARRIER IN IBD

Defects in the intestinal epithelial barrier function are a characteristic feature of IBD. The perturbations can affect different levels of the protective mechanisms, including alterations of PRRs, disturbed AMP production, a defective mucus layer, alterations in the process of autophagy or an increased epithelial barrier permeability, and cause an inadequate protection against microbial adherence and invasion. The resulting imbalance between

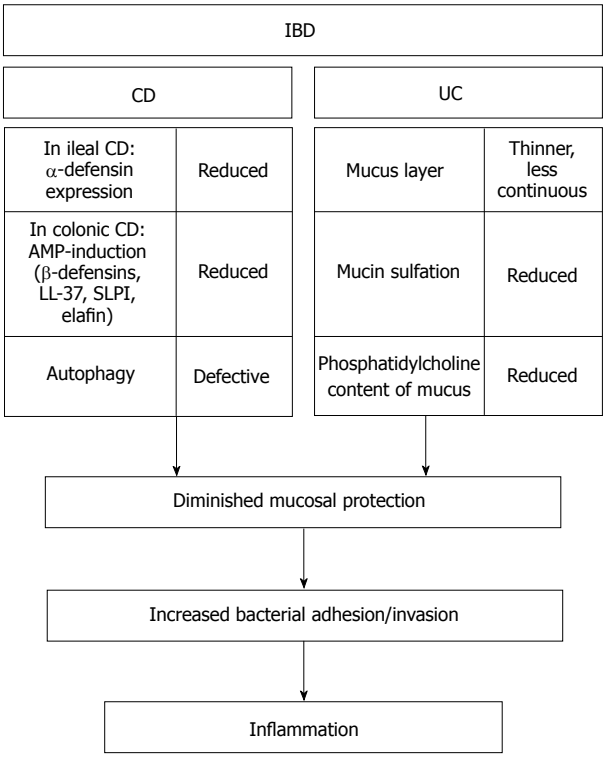

Figure 2 Defects of the intestinal mucosal barrier in inflammatory bowel disease and their consequences. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

the microbes and the protective host-defense mechanisms allow more bacteria to come in direct contact with the epithelium and mucosal immune mechanisms. Consequently, compensatory immune reactions are excessively triggered, a process that is thought to finally result in chronic intestinal inflammation (Figure 2).

As IBD affects predominantly the most heavily colonized gut segments, namely the distal ileum and colon, an involvement of the microbial flora in disease pathogenesis is obvious[5]. This is also indicated by studies showing that postoperative recurrence of Crohn's disease (CD) is triggered by luminal contents[83,84]. Moreover, several animal models revealed the essential contribution of microbes to the development of inflammation[85,86]. Yet, despite extensive research, no specific IBD-causing pathogen could be identified until now[87,88]. Instead, the intestinal microbial community as a whole appears to be crucially involved in disease pathogenesis[89]. Studies showing a marked increase in surface associated bacteria and to some extent also intracellular bacteria in patients with IBD confirm this assumption[90-92]. This suggests a deficiency in the protective mechanisms that effectively keep away microbes from the epithelial surface in the healthy intestine. In the following sections, important aspects of barrier perturbations in IBD will be discussed in more detail.

Pattern-recognition receptors and antimicrobial peptides

In 2001, different groups identified independently the NLR-family member nucleotide-binding oligomerization domain-containing protein 2/Caspase recruitment domain-containing protein 15 (NOD2/CARD15) as a susceptibility gene for CD[93,94]. There are three common genetic variants associated with CD, two missense mutations and one insertion frameshift mutation that leads to a truncation of the mature NOD2 protein[4,20]. As Kobayashi *et al*[95] showed in a study on NOD2$^{-/-}$ mice, NOD2 plays a pivotal role in the intestinal host-defense. The NOD2-deficient mice were highly susceptible to intragastric challenge with *Listeria monocytogenes* but not to intraperitoneal or intravenous infection and displayed reduced levels of a subset of Paneth cell α-defensins. Furthermore, the important role of NOD2 in intestinal homeostasis was underscored by the observation that NOD2 is crucially involved in the regulation of the intestinal flora. Alterations in the composition and quantity of the commensal microbiota were observed in NOD2-deficient mice[96]. Interestingly, CD patients homozygous for the NOD2 variant SNP13 displayed similar changes that were characterized by a significantly increased load of Bacteroidetes and Firmicutes in their terminal ilea[96]. Moreover, Petnicki-Ocwieja *et al*[97] detected increased amounts of commensal bacteria in the terminal ilea, a diminished capability to prevent *de novo* colonization of the opportunistic pathogen *Helicobacter hepaticus* and a reduced antimicrobial activity of crypt secretions in NOD2-deficient mice. Notably, an interrelation of NOD2, antimicrobial peptide expression and intestinal defense in humans was emphasized by a study showing reduced levels of Paneth cell α-defensins in patients with ileal CD that was accompanied by a reduced antibacterial activity of protein extracts from ileal mucosa. Interestingly, the defensin reduction was especially pronounced in patients carrying the mutation NOD2-SNP13[98]. These findings highlight the important role of a diminished antibacterial host-defense as a primary factor in disease pathogenesis.

Recently, a second member of the NLR-family, namely NLR family, pyrin domain containing 3 (NLRP3), has been implicated in the pathogenesis of IBD. NLRP3 is as a constituent of the inflammasome involved in the activation of caspase-1 and the subsequent maturation of interleukin-1β and interleukin-18[99,100]. Villani *et al*[101] reported an association between SNPs (Single nucleotide polymorphisms) located in a predicted regulatory region downstream of NLRP3 and an increased risk to develop CD and Schoultz *et al*[102] suggested a role for combined polymorphisms in CARD8 and NLRP3 in the development of CD in men but an implication of the NLRP3-region in CD-susceptibility was not confirmed in a recent study[103]. Thus, the available data in this context are inconsistent and further studies are required.

However, studies on NLRP3$^{-/-}$ mice showed that NLRP3 plays a central role in the regulation of intestinal homeostasis and prevention of colitis. Zaki *et al*[104] studied NLRP3$^{-/-}$ mice and showed that these mice were significantly more susceptible to DSS-induced colitis compared to wild-type mice. A clearly increased susceptibility of NLRP3$^{-/-}$ mice to experimental colitis was also report-

ed by Hirota *et al*[99] whereas, in contrast to these studies, Bauer *et al*[105] found that NLRP3-deficient mice were significantly protected from colitis in the DSS-colitis model. Interestingly, in the study of Hirota *et al*[99] the NLRP3$^{-/-}$ mice displayed also an altered colonic β-defensin expression, a reduced antimicrobial activity of colonic crypt secretions and an intestinal dysbiosis. These results are of special interest with regard to the pathogenesis of IBD as a dysregulated β-defensin expression has been linked to the development of colonic CD. Macroscopically and histologically non-inflamed tissue samples of CD-patients with colonic involvement displayed reduced mRNA-levels of the constitutively expressed β-defensin HBD-1[106]. Moreover, defects in the expression of the inducible β-defensin HBD-2 were reported. In ulcerative colitis (UC)-patients, the expression of this β-defensin is strongly induced in the case of inflammation but this induction is diminished in patients with colonic CD[39] and the expression of two other β-defensins, HBD-3 and HBD-4[37,39] as well as the cathelicidin LL-37[107] and the antimicrobially active protease inhibitors SLPI and elafin[108] seems to follow a similar pattern. The functional relevance of these findings was pointed out by a study showing a reduced antimicrobial activity of colonic tissue protein extracts in colonic CD patients[109].

Moreover, polymorphisms in the genes of several members of the TLR-family have also been associated with IBD[11] and especially extensively investigated was the influence of TLR4-ploymorphisms on the risk to develop CD. Whereas some studies showed a correlation between TLR4 genetic variants and CD[110-112], in other studies no such association was found[113-115]. Thus, the available data in this regard are contradictory and no definite conclusion can be drawn to date.

PANETH CELLS AND GOBLET CELLS

Wnt-pathway

As NOD2 and α-defensins are both associated with small intestinal Paneth cells, it was apparent that defects in Paneth cell biology play a crucial role in small intestinal CD. Thus, further studies were conducted to characterize the role of Paneth cell-dysfunction and diminished antimicrobial host-defense in CD in more detail and it was shown that the Wnt-pathway transcription factor T-cell specific transcription factor 4 (TCF-4, also known as TCF7L2, transcription factor 7-like 2) is critically involved in this context. Patients with ileal CD displayed, independent of the degree of inflammation, a reduction in TCF-4 mRNA-expression and the TCF-4-mRNA-levels correlated highly with the mRNA-levels of the Paneth cell α-defensins. Importantly, the association between α-defensins and TCF-4 was independent of the NOD2-genotype. Additionally, a causal link between reduced TCF-4- and reduced α-defensin-levels was demonstrated by a TCF-4-knock out mouse model in which TCF-4-heterozygous mice displayed a significantly reduced α-defensin expression that was associated with a reduced antibacterial activity of small intestinal tissue extracts[116]. Moreover, in a later study an association between a sequence polymorphism in the TCF-4 putative promoter region and a higher susceptibility to develop small intestinal CD was reported[117]. Recently, it was uncovered that another central factor of the Wnt-pathway, the co-receptor low density lipoprotein receptor-related protein 6 (LRP6), is required for proper antimicrobial Paneth cell function and is involved in the development of CD. More specifically, a functional variant in the Wnt co-receptor (rs2302685; Ile1062Val) has been associated with early onset ileal CD and, importantly, patients carrying this variant were shown to have particularly low Paneth cell α-defensin-levels. Moreover, the LRP6-mRNA-levels were generally reduced in ileal CD independently of the genotype[118]. In conclusion, the link between genetic variants in crucial components of the Wnt-pathway, diminished α-defensin levels and ileal CD indicate that the diminished Paneth cell antimicrobial function is a primary pathogenic factor in CD. Moreover, since the Wnt-pathway does not only directly regulate α-defensin expression but also influences Paneth cell maturation, these data suggest that an impaired Paneth cell differentiation may be involved in CD pathogenesis[13,119].

ER-stress and the unfolded protein response

In recent years, unresolved ER-stress and the unfolded protein response (UPR) have repeatedly been implicated in the pathogenesis of CD as well as UC. A first indication of a relation between ER-stress and intestinal inflammation was provided by a study from 2001 that revealed an increased susceptibility to DSS-induced colitis in mice deficient in the ER-stress transducer IRE1β[120]. In a more recent study, deletion of another key component of the ER-stress response, the transcription factor X-box-binding protein 1 (XBP1), in the intestinal epithelium of mice lead to increased ER-stress, spontaneous small intestinal inflammation and also increased sensitivity towards induced colitis. Interestingly, Paneth cells were absent in these mice and the number of goblet cells in the small intestine was also reduced. As Paneth cells normally secrete large amounts of antimicrobial molecules, the Paneth cell depletion resulted in a reduced antibacterial activity of crypt supernatants as well as an increased susceptibility to oral infection with *Listeria monocytogenes*. Importantly, a possible link between XBP1 and human disease was provided by the detection of an association between genetic variants of XBP1 and IBD[121]. Moreover, another clear indication that the process of ER-stress may be implicated in IBD pathogenesis gave the detection of an overexpression of the ER-localized stress response chaperone Gp96 on the apical surface of ileal epithelial cells in patients with CD. This chaperone is used as a receptor for adherent-invasive *Escherichia coli* (*E. coli*) invasion *via* outer membrane vesicles (OMVs) rich in the outer membrane protein OmpA[122]. These are

especially interesting findings with regard to the older observation that adherent-invasive *E. coli* strains are highly associated with the ileal mucosa in CD[123-125].

Furthermore, particularly noteworthy in the context of ER-stress and IBD pathogenesis are several studies that provide a link between ER-stress, defective mucus production and susceptibility to intestinal inflammation. Heazlewood *et al*[126] generated and characterized two mice-strains, called Winnie and Eeyore, each with a single missense mutation in the *MUC2* gene. These mice developed spontaneous intestinal inflammation and showed a phenotype reminiscent of human UC. Moreover, they were more susceptible to environmentally induced colitis and showed profound changes in the process of MUC2 synthesis and assembly. This went along with an abnormal mucin distribution in goblet cells - the goblet cell theca were smaller and reduced in number and a cytoplasmic accumulation of the non-*O*-glycosylated MUC2-precursor in vacuolar structures was detectable. Importantly, goblet cells showed ultrastructural and biochemical evidence of ER-stress and activation of the UPR. Furthermore, in tissue samples of UC patients a cytoplasmic MUC2-precursor accumulation was also detectable and this coincided with signs of protein misfolding and ER-stress. From these findings the authors concluded that ER-stress related changes in mucin production could be crucially involved in the pathogenesis of UC.

Also linked to mucus production, the ER-stress response and prevention of intestinal inflammation is anterior gradient homolog 2 (AGR2), a member of the family of protein disulfide isomerases that is present in the ER of intestinal secretory epithelial cells. Recently, AGR2 has been shown to play an essential role in intestinal mucus production and in this context, a direct association of AGR2 and MUC2 *via* the formation of mixed disulfide bonds between a cysteine in the thioredoxin-like domain of AGR2 and cysteines in the amino- and carboxyl-terminal portions of MUC2 are thought to be crucial. The importance of AGR2 for mucus production was demonstrated in AGR2$^{-/-}$ mice that lacked intestinal mucus and MUC2 protein and showed increased susceptibility to DSS-induced colitis. Although the mice developed no overt spontaneous inflammation, they displayed an increase in the expression of several proinflammatory cytokines and in the presence of intestinal mast cells[127]. In a more recent study, AGR2$^{-/-}$ mice generated by Zhao *et al*[128] displayed severe spontaneous terminal ileitis and colitis. Moreover, the mice lacked morphologically normal goblet cells and showed decreased MUC2 protein levels. Interestingly, the intestinal disturbances were not restricted to goblet cells as severe Paneth cell abnormalities were also detectable including a dramatic expansion of the Paneth cell compartment and an abnormal Paneth cell localization. As the Paneth cell expansion preceded the occurrence of inflammation it was not a secondary phenomenon resulting from inflammatory processes. Whereas Park *et al*[127] reported only a modest evidence for activation of the ER-stress response, Zhao *et al*[128] detected clearly elevated levels of intestinal ER-stress. Albeit the definite cause is to date not clear, the discrepancies between the studies of Park *et al*[127] and Zhao *et al*[128] were attributed to differences in the genetic background and experimental design as well as to diverging housing conditions. In conclusion, the mentioned AGR2$^{-/-}$ mouse models underline the importance of AGR2 in the intestinal goblet cell- as well as Paneth cell-homeostasis and the ER-stress response and are particularly interesting since genetic variants of AGR2 and decreased AGR2-mRNA-levels have been linked to IBD[129].

Autophagy

Autophagy is a degradation process important for cellular homeostasis that allows the cell to recycle cellular components by delivering organelles and cytosolic macromolecules to lysosomes. The process is activated in response to situations of cellular stress like starvation and growth factor deprivation. Moreover, the autophagy machinery is also important in the antibacterial host-defense as it is involved in the degradation of invading bacteria[130-132].

Recently, a defect in the process of autophagy has been linked to the pathogenesis of CD as a polymorphism in the gene encoding the central autophagy protein autophagy-related 16-like 1 (ATG16L1) has been associated with an increased risk to develop the disease[133]. Moreover, a second autophagy-related protein, immunity-related GTPase family M protein (IRGM), has been related to CD[131,134,135]. In subsequent studies, the role of ATG16L1 in CD-pathogenesis has been investigated in more detail and it was reported that Paneth cells of ATG16L1-hypomorphic mice displayed abnormalities in the granule exocytosis pathway. Importantly, homozygous carriers of the disease associated risk allele of ATG16L1 among CD patients exhibited similar changes in Paneth cell granules[136]. Additionally, Kuballa *et al*[130] demonstrated in cell culture experiments that the CD-associated ATG16L1 coding variant (ATG16L1*300A) is linked to a defective anti-Salmonella autophagy. Furthermore, a link between the process of autophagy and CD was provided by a study on paediatric CD patients that revealed an activation of autophagy in Paneth cells that occurred independently of inflammation or disease-associated variants in ATG16L1 or IRGM and went along with a significant reduction in the number of secretory granules and signs of crinophagy[137]. Remarkably, in a recent study the intracellular PRRs NOD1 and NOD2 have been shown to be involved in the induction of autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. As the CD-associated NOD2 mutant L1007fsinsC was defective in this process, these findings provide a mechanistic link between two of the most important genes associated with CD, NOD2 and ATG16L1, and highlight the relevance of defects in the process of bacterial sensing and elimination in the disease pathogenesis[138].

ALTERATIONS OF THE MUCUS LAYER

Variations of the intestinal mucus layer concerning its thickness, continuity and composition as well as the mucin-structure have been reported in IBD patients, especially in UC. These changes are thought to adversely affect the protective properties of the gel layer and consequently might cause an increased vulnerability of the epithelium to bacterial invasion.

The mucus layer has been shown to be thinner and less continuous in UC patients[139], its phosphatidylcholine concentration is significantly reduced[73] and, in some older studies, a selective reduction of a specific mucin glycoprotein fraction in the colonic mucosa has been reported[140,141]. However, as the latter results could not be reproduced in a subsequent investigation[142], they were interpreted as an effect of the experimental design and were thought to rather reflect the general mucus depletion characteristic for UC patients[142,143]. Furthermore, an altered mucin glycosylation was detected in UC patients - the glycans are shorter, have a generally less complex structure[144,145] and their sulfation is reduced[64,146]. As the sulfates confer negative charge to the mucins and enhance the resistance of the glycans against enzymatic degradation, a reduced sulfation could result in an increased vulnerability of the mucins to bacterial enzymatic degradation[64,66,146] and, since positively charged AMPs are electrostatically bound by mucins[80,81], might cause a less effective retention of AMPs in the mucus layer. Whether the reported changes are primary events in disease pathogenesis or are secondary effects resulting from inflammation is currently under discussion. Whereas some studies indicated that mucus or mucin alterations occur only in active UC[144,147], other studies gave evidence of primary alterations as they revealed an ethnic and genetic impact[141,148-150]. Recently, mucus depletion in UC has been linked to a defect in goblet cell differentiation as the significant induction of the goblet cell differentiation factors Hath1 and KLF4 seen in CD patients in inflammation was not detectable in UC patients and, moreover, a reduced number of mature goblet cells in the upper third of the crypts in sigma tissue of patients with active UC was observed[151].

EPITHELIAL PERMEABILITY

Already in the early 1980s, increased intestinal permeability has been reported in children with active small-bowel CD[152] and, more recently, increased permeability has also been described in UC patients[153,154]. Several studies observed increased permeability not only in CD patients but also in a subset of their first-degree relatives, leading to the assumption that increased permeability may be a primary factor in disease pathogenesis preceding the development of inflammation[155-157]. Interestingly, in a recent study an association between a high mucosal permeability in healthy first-degree relatives of CD patients with the presence of a CARD15 3020insC-mutation has been observed[158]. Moreover, increased permeability has been linked to a higher risk of disease relapse in CD patients[159,160], suggesting that the permeability increase precedes clinically overt inflammation. However, in other studies a permeability increase was also detected in a proportion of spouses of CD patients[161-163], an observation that indicates an impact of environmental factors on permeability alterations. To date, it is still under debate whether the permeability changes are a primary event in the disease development or a secondary effect triggered by inflammation. The perturbations of intestinal permeability are assigned to different molecular mechanisms including tight junction abnormalities[8,15,164]. For example, in a recent study a reduced number of tight junction strands and an increased number of strand breaks were observed in CD patients[165]. Moreover, an increased expression of the pore-forming claudin 2 and a decreased expression of occluding and the sealing claudins 5 and 8 was detectable. These changes were observed in patients with mild to moderately active CD but were absent from patients in remission indicating that they were a rather secondary phenomenon occurring as a consequence of inflammation[165]. In UC claudin 2 was also upregulated, whereas occludin and the claudins 1 and 4 were diminished[166].

Additionally epithelial apoptosis also results in an increased epithelial permeability.

Several studies showed that in inflammatory bowel disease apoptosis is upregulated. Sipos *et al*[167] found the rates of epithelial apoptosis determined by the histological activity of inflammation in UC. IL-13 triggers an increased epithelial apoptosis rate that leads to microerosions[166] in an early stage of the disease. In contrast, in active CD apoptosis is also upregulated[166,168], but microerosions are only induced in advanced disease.

Using confocal laser endomicroscopy, Goetz *et al*[169] found a higher density of epithelial gaps in the mucosa of patients with CD. In a mouse model TNF led to an elevated epithelial cell shedding and increased the gap density[170].

In conclusion, epithelial permeability perturbations occur in IBD patients but additional investigations are required to further increase the knowledge about the underlying molecular mechanisms as well as to clarify whether these changes play a primary role in disease pathogenesis or whether they are a secondary effect in response to inflammation. It is important to note that carbohydrates such as lactulose and mannitol, the low molecular weight polyethylene glycol PEG 400 or ^{51}Cr-labelled ethylenediaminetetraacetic acid were used as probe molecules to measure the permeability of the intestinal wall in the mentioned studies. As increased permeability for these substances does not give information on epithelial permeability for bacteria and bacterial products, it is not possible to deduce that the described permeability changes are associated with a defective protection of the mucosa against luminal microorganisms and an increased exposure of mucosal immune mechanisms to microbial

antigens.

CONCLUSION

The healthy intestinal mucosa is efficiently protected against detrimental influences from the gut lumen by a complex and multilayered defense barrier. In recent years, it became apparent that the intestinal barrier is compromised at different levels in IBD patients. These barrier disturbances include alterations of the mucus layer, a defective AMP production, unresolved ER-stress, defects in the process of autophagy as well as an increased epithelial permeability and result in an inadequate protection of the epithelium against adherence and invasion of luminal bacteria. Consequently, abnormal high numbers of microorganisms are allowed to come in direct contact with the hosts immune mechanisms and this is thought to initiate a process finally resulting in chronic intestinal inflammation.

Future studies are required to further increase our understanding of the complex mechanisms underlying the pathogenesis of IBD, to clarify their complex interplay and to enable the development of new therapeutic approaches for the treatment of IBD.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Magnetic resonance imaging in children and adolescents with chronic inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBD) represent challenges, both from a diagnostic, and therapeutic point of view. Deep-seated anatomic structures are difficult to assess by ultrasound technique alone. As radiation-free alternative cross-sectional imaging method, magnetic resonance imaging of the intestinal structures is costly and time-consuming. Examination of pediatric patients imply additional considerations: reduction of body motions in younger children and consideration of the most appropriate preparation, and examination technique. The demanding Sellink technique is the only means for appropriately distending the lesser intestine in order to detect small bowel strictures. Oral intake of contrast medium (CM) alone shows its limitations regarding distensibility. The need for intravenous contrast media application needs to be considered, too. Active inflammation of both intestinal wall, and mesentery can be demonstrated accurately. Nevertheless, viable alternatives to CM application is desirable, considering non-negligible adverse reactions. Recent data suggest diffusion weighted imaging might fill this diagnostic gap. Irrespective of sequence technique chosen, bowel movement remains a major obstacle. Antispasmolytics in their function as smooth muscle relaxants help in improving image quality, however, their use in children might be off-label. Optimal preparation for the examination and appropriate imaging technique allow for diagnosing typical patterns of changes in IBD, such as bowel wall thickening, ulcers, mural stratification, strictures, creeping fat, and comb sign, and lymphadenopathy. The article gives a detailed overview of current significance of magnetic resonance imaging pediatric patients suffering from IBD, considering indications, limitations, and safety aspects.

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Key words: Children; Adolescents; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Magnetic resonance imaging; Enterography

Core tip: Diagnosis of chronic inflammatory bowel disease (IBD) is partially based on subsequent imaging. Magnetic resonance imaging (MRI) of the gastrointestinal tract (GIT) is established in adults for diagnosing IBD. In children and adolescents MRI is not routinely used up to now. This manuscript presents the commonly used magnetic resonance sequences for the evaluation of the GIT in children and adolescents. Techniques to obtain optimal bowel distension by oral intake or by using a nasally placed tube are described. Typical findings of intestinal and mesentery pathology in children suffering from IBD are shown.

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imaging in children and adolescents with chronic inflammatory bowel disease. *World J Gastroenterol* 2014; 20(5): 1180-1191 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1180.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1180

INTRODUCTION

Population-based studies suggest that inflammatory bowel disease (IBD) is unevenly distributed throughout the world with the highest incidence rate occurring in the Western world. However, emerging data have demonstrated a rising trend of IBD in Eastern countries[1]. In approximately 20%-30% of all affected patients with IBD the disease manifests in childhood[2]. Studies have shown a prevalence of as high as 16.6 per 100000 in the pediatric population[3]. Chronic IBD, namely Crohn's disease (CD) and ulcerative colitis (UC) seem to develop as a result of dysregulation of the immune response to normal gut flora in a genetically susceptible host[4] (Table 1). There are further reasons, like microvascular, infectious, and environmental co-factors to consider[5,6]. Moreover changes in lifestyle and diet, migration-related changes in genetic susceptibility, urbanization, and environmental changes may be relevant cofactors. Epidemiologic data indicate an increase in IBD in children. While CD and UC occur with equal distribution in adults, there are three new CD cases for each new UC case in pediatric age groups[7]. There are patients who, however, can't be classified as CD or UC. They are termed inflammatory bowel disease-undeterminate (IBD-U). In the pediatric population a greater number of IBD are labeled as IBD-U compared to adults. In adult IBD there is an equal ratio of male to female patients, or perhaps more women affected by the disease. In contrast, prepubertal males seem to be more affected than females, with a male predominance of 1.5:1[8]. Rarely, UC can even be found in young children and infants. In children an overlap of clinical findings can be observed, making differentiation of both entities in the pediatric patient difficult. IBD in children often relapses, making repeated imaging necessary.

CD can affect any part of the gastrointestinal (GI) tract, however, in children the distribution tends to be more proximal. The terminal ileum is the most common site of CD; however about 60% of pediatric patients show ileocolonic involvement, whereas 20%-30% have isolated colonic disease[1]. Transmural spread is a distinguishing feature of CD. Up to one third of patients will develop a perianal fistula or abscess at some point in their disease course. Skip lesions, fibrofatty proliferations, and mesenteric lymphadenopathy are common. In the case of chronification, fibrosis of the bowel wall may occur, resulting in stenotic bowel loops.

UC is mostly localized to the colon with the rectum affected primarily. Even if pancolitis is the most common presentation in childhood other features exist[9]. UC is a chronic inflammation involving exclusively the mucosa of the colon. Diarrhea and rectal bleeding is seen commonly in UC (50%-90% of cases)[10]. Contrary, perianal or perirectal disease is not a feature of UC.

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

Diagnosis of IBD is based on clinical presentation, laboratory and endoscopic assessments, histology and subsequent imaging[11,12]. Proper classification is necessary in order to determine the ideal treatment in children with chronic disease. The Montreal classification was adapted to the pediatric population[13]. In a child with bloody diarrhea a bacterial infection needs to be excluded first. Further differential diagnosis includes vasculitis, ischemic colitis, and hemolytic uremic syndrome. Bleeding without diarrhea is possible in children with fissure, vascular malformations, coagulation disorders, polyps, or Meckel diverticulum. Etiologies that can mimic IBD include appendicitis. Moreover, food allergies like cow's milk protein allergy can result in GI bleeding as early as in infancy. Laboratory studies can help to identify a child suspected to suffer from IBD and to monitor the course of the disease. Diagnostic imaging is necessary to describe the extension and grading of the inflammation. In children, ultrasound can be used with high success. The use of high frequency transducers (7-14 MHz) allows a detailed assessment of the whole bowel. Graded compression allows for displacement of adjacent bowel loops and interfering intraluminal gas. Color Doppler can be used to assess inflammation by demonstrating increased perfusion and vascularization. Significant correlations could be shown by considering bowel wall thickness, vascular pattern, and disease activity. Ultrasound can be used to appreciate extraintestinal findings (lymph node enlargement, abscesses), and to describe the surrounding mesenteric tissue. On the other side, ultrasound can fail to detect IBD in cases of superficial disease or if there are artifacts (*e.g.*, bowel gas). Also, ultrasound is an operator dependent modality; experience is necessary in order to make the correct diagnosis. Plain radiography is reserved for acute diseases. Findings are nonspecific and can include large bowel dilation, and small bowel distension. In the acute abdomen, the toxic megacolon or obstruction can be suspected. These cases have to be followed in order to detect possible bowel perforation. Contrast enema can be helpful for the evaluation of the extension of the disease, moreover, it is helpful to rule out or prove stenosis. Upper gastrointestinal series with small bowel barium follow through (SBFT) has been the cornerstone of small bowel imaging in the past[14]. The more sensitive enteroclysma was considered the gold standard (so-called Sellink technique), but it is not easy to perform, especially in younger children and infants. It requires the insertion of a naso-jejunal tube, for that it is poorly tolerated by children. It comes with a considerable higher radiation dose compared to SBFT[15]. Both methods suffer from one further major disadvantage: its

Table 1 Inflammatory bowel disease in children

	Crohn's disease	Ulcerative colitis
Etiology	Unknown (hereditary, immune response to gut flora)	
Incidence		
Site	Terminal ileum/SB/entire GIT	Colon/backwash ileitis
Pattern	Skip lesions, transmural, deep ulcerations	Diffuse mucosal
Acute complication	Obstruction, toxicity, hemorrhage, perforation	
Chronic complication	Stenosis, fistula, fissures, abscess, GIT cancer, extraintestinal manifestation	Extraintestinal manifestation, colorectal cancer

SB: Small bowel; GIT: Gastrointestinal tract; PSC: Primary sclerosing cholangitis.

very limited information regarding the extraluminal mesenteric extension of the disease. Computed tomography (CT) as a cross-sectional imaging method overcomes this and may be helpful in the acute situation, for planning surgery, especially in the evaluation of stricture or obstruction. Again, CT enteroclysma is poorly tolerated in children[16]. The major disadvantage of CT is the large amount of radiation exposure[17]. So, especially in children, techniques using ionizing radiation should be avoided if possible[18]. Leucocyte scintigraphy using white blood cells labeled with technetium 99m, and also positron emission tomography may be indicative in IBD, however, these techniques are not usually used. Because IBD is a diagnosis which should be proven by endoscopy with biopsy and histologic assessment, they are part of the diagnostic workflow. Endoscopy is the mainstay for evaluation, but provides limited access to the small bowel (terminal ileum and duodenum) and can be more limited in the presence of severe disease or strictures. Capsule endoscopy can overcome parts of these limitations, but has limitations as well especially in stricturing disease. Recent advances in magnetic resonance imaging (MRI) have the potential to emerge as useful imaging technique providing assessment of the whole gastrointestinal tract and to assess extra-intestinal involvement without ionizing radiation.

In adults MRI of the GI tract is now an established method when diagnosing chronic inflammatory bowel disease. In children and adolescents MRI is not routinely used, but some guidelines define MR as first line imaging modality in the investigation of the GI tract in suspected chronic inflammatory bowel disease. Especially the lack of radiation burden would favor the use of MRI. Further advantages include the superior soft tissue contrast and multiplanar imaging. Improvements in MR hardware include the availability of fast breath-hold sequences, decreased scan time and increased spatial resolution.

But, there are some difficulties of the technique in infants that limit the use of MRI. Children at school age have no problems with undergoing MR study, but the younger ones need to be handled gently because of reduced compliance. Numerous articles have been published on this topic discussing the complexity and also the financial impact on management. In the literature only few scientific reports and reviews on MRI of the GI tract in children can be found[19]. The purpose of this article is to present the commonly used MR sequences in children for the evaluation of the GI tract in the diagnosis and follow-up of IBD.

MR IMAGING OF THE BOWEL

Currently, there is no approved standard protocol for the evaluation of IBD using MRI. Applying a standard protocol without bowel distension is not helpful and results in false negative findings. The use of spasmolytics such as n-butylscopolamine (hyoscine) (0.5 mg/kg, up to 20 mg)[20] or glucagon[21] might meet the criteria of off-label use in some countries. Intravenous application of spasmolytics is given immediately before the start of the MR scans. A further bolus can be administered immediately before contrast media application. Metoclopramid (off-label in some countries) (0.15 mg/kg) can be given in the case of nausea. The cleansing preparation of the small bowel consists of a low-residue diet starting 3 d prior to the examination (no milk products, ample fluids, non-carbohydrate in order to avoid the generation of gas).

The cardinal principle to obtain diagnostic images is an optimal bowel distension of the bowel lumen. The loops need to be distended in order to detect changes in luminal diameter, which can be observed in cases with relative bowel stenosis. However, there is no consensus about the technique for optimal small-bowel distension[22]. Commonly used agents include methylcellulose, locust-bean gum, psyllium, and mannitol. There are very different protocols in the literature, ranging from oral application before entering the MR cabin (hydro MRI) to the MR enteroclysma procedure (MR Sellink) using application of methylcellulose while in the MR scanner *via* a nasojejunal tube.

Oral intake of endoluminal agents is mandatory. Some studies have shown, that oral intake is sufficient and increases the patient's compliance (which is necessary especially in follow-up studies)[23]. Unfortunately, sometimes the filling is inadequate and the distal/terminal ileum is not sufficiently distended (Figure 1). The most accurate results can be achieved with the MR enteroclysma technique[24]. Reports in the literature describe numerous different oral contrast media suitable for use, including plain water, mannitol, sorbitol, iron oxide, and barium sulfate, ispaghula husk, locust bean gum, planta ovate, and pineapple juice[25]. Based on their signal intensity in T1 weighted (T1w) and in T2 weighted (T2w) imaging, these contrast substances are divided

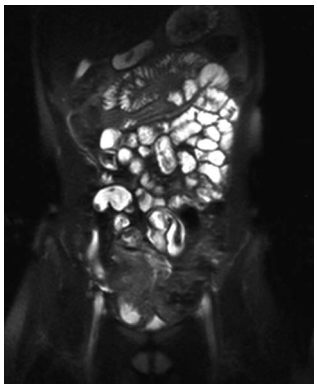

Figure 1 Hydro-magnetic resonance imaging using mannitol orally. Sometimes the filling result is inadequate and the distension of the bowel is insufficient.

into so-called positive (hyperintense in T1w and in T2w sequences) and negative (hypointense in T1w and in T2w sequences) contrast media. Some authors use a combination of barium sulfate and sorbitol as described by Sauer *et al*[26] who administered 450 cm^3 of 0.1% barium sulfate with sorbitol 90 min before imaging and a second amount of 450 cm^3 30 min before imaging[1]. Dagia *et al*[27] administered sorbitol orally (1000-1500 mL) as enteral contrast agent by adding 15 mL of sorbitol to 500 mL of water. Application started 60 min before the scan at a rate of 250-300 mL/15 min. If distension of the proximal small bowel was inadequate on initial MR acquisition (luminal diameter < 3 cm), another 250-300 mL of sorbitol was given[27]. Others use a suspension of polyethylene glycol (PEG; Klean-Prep®, Helsinn Birex Pharmaceuticals Ltd.) one hour before the investigation (1500 mL) after an overnight fasting state[20]. To render these mixtures more palatable, small amounts of orange flavoring may be added[3]. Mannitol and other hypertonic contrast agents may have side-effects, such as excessive diarrhea, which is an important issue, especially in children. The influence of the osmolarity for small bowel distension could be shown in a study by Ajaj *et al*[25] who compared a water solution combined with 2.0% sorbitol and 0.2% locust bean gum (quantity 1500 mL, osmolarity 1148 mOsmol/L) with a solution combined with 2.0% sorbitol and 2.0% barium sulfate (quantity 1000 mL; osmolarity 194 mOsmol/L). The mean loop diameter after solution administration with higher osmolarity increased over time (up to 30 min). The side effect rate of both solutions was low, but the smaller amount was more acceptable for patients[28]. Alexopoulou *et al*[29] used a total amount of 2.5 L of water solution containing 0.1 g/kg body weight of herbal fibres (psyllium) which was administered orally over a period of 4 h before MRI. This mixture has the property of retaining large amounts of water, up to 20 to 30 times its own volume, thus providing adequate distension. This agent also has biphasic properties, demonstrating low signal intensity in T1w images and high signal intensity in T2w images. MR enteroclysma-which the authors prefer-allows for dynamic filling of the small bowel. Thereby, relevant stenosis can be detected dynamically. In this procedure, the patients will be provided with a tube inserted nasally, which will then be proceeded past the ligament of Treitz under fluoroscopic guidance, using a reduced pulse rate (rate 3 pulses/s; fluoroscopy time 1-3 min[27]; authors' own experience: 4 s-1 min; Figure 2). The procedure of inserting and positioning of the catheter is the most challenging part of the entire procedure and can become a very unpleasant or almost intolerable maneuver for both the patient and the radiologist[30]. Approximately 1000-1500 mL prewarmed methylcellulose will be administered manually during the MR scanning process over a time period of up to 15 min using 50 mL syringes. The filling can be automated by a pump which usually needs to be placed outside of the scanner room. The filling process will be followed by using a single-shot heavily T2w sequence with thick slabs (7-10 cm) in coronal orientation which are sequentially acquired during continuous filling of the bowel (Figure 3). This dynamic acquisition results in a film sequence of the filling process which can be helpful in the detection of atonic bowel segments, and hyperperistalsis, or in the delineation of stenosis and luminal diameter changes. If the patient suffers from nausea, the filling process needs to be stopped and may be continued after recovery. The total amount of methylcellulose tolerated is limited by patient compliance and by the amount of backflow of cellulose into the duodenum and the stomach. Filling is finished if the terminal ileum is widened and the coecum/ascending colon start to become distended. Necessity of additional placement of a transrectal catheter and filling of the colon depends on the region of suspected pathology[31].

During the MR procedure the patients may be placed in the prone position, for a mild pressure to the anterior abdominal wall may result in better separation of the small-bowel loops. In case of MR enteroclysma this position will not be tolerated well. So, the authors suggest that the patient is placed in the standard supine position. In case of an unsuccessful separation of the loops the patient can be placed in the prone position at the end of the MR examination.

For MRI a dedicated multi-channel body array coil is used. The main technique involves MRI with ultrafast T2w sequences supplemented by dynamic contrast enhanced T1w scans in combination with fat saturation covering abdomen and pelvis, from the lung bases to the perineum. True steady-state free precession images (TRUFI) are obtained in the coronal plane for overview. T2w half-fourier acquisition single-shot turbo-spin-echo (HASTE) imaging is performed with long time of echo in axial and coronal orientation. T2w sequences are obtained with and without fat saturation. Fast low-angle shot T1w sequences (FLASH) are acquired in axial orientation. For dynamic multiphase contrast studies the authors use 3D sequences (T1-volume interpolated gradient-echo = VIBE) (Figure 4). Gadolinium-based agent at a dose of 0.1 mmol/kg will be administered intravenously. T1w VIBE sequences are acquired imme-

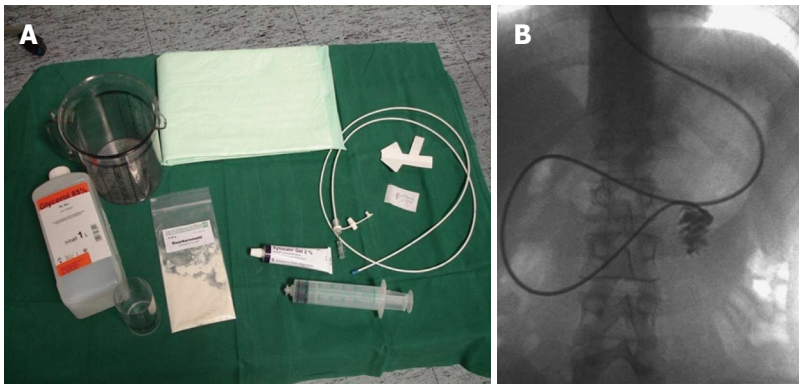

Figure 2 Magnetic resonance enteroclysma requires application of a nasojejunal tube (A) and placement will be guided by pulsed fluoroscopy (B).

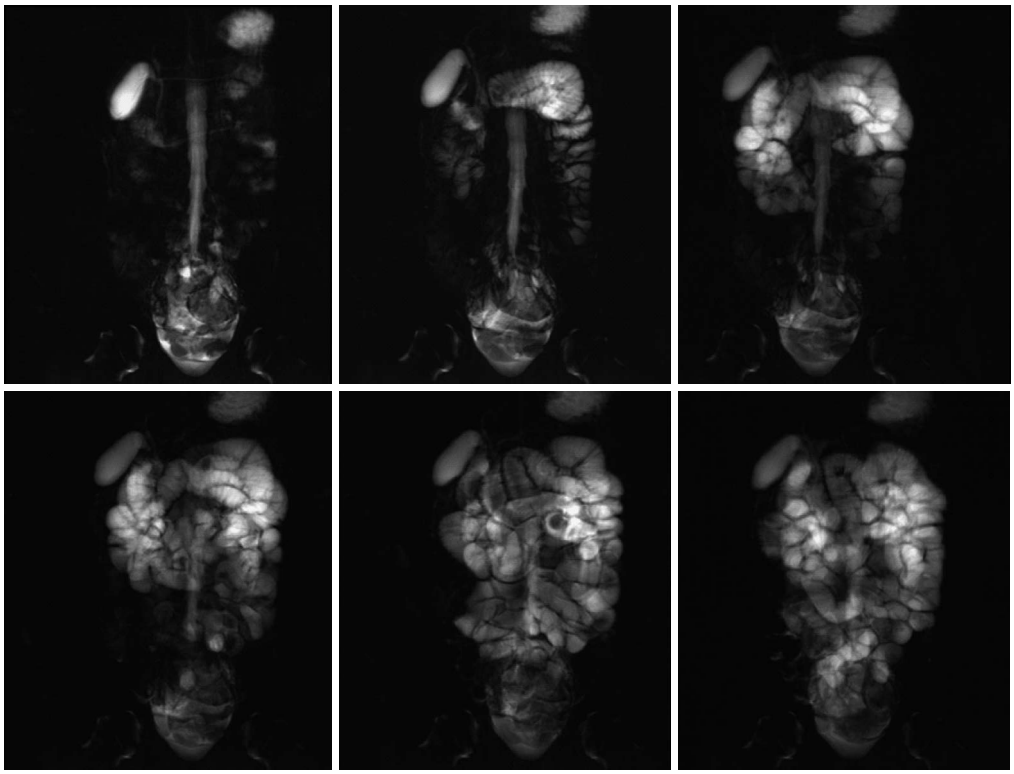

Figure 3 Enteral filling can be followed by using thick slab T2 weighted sequence (7-10 cm thickness).

diately after contrast application and repeated 30, 60, 90, and 180 s post injectionem (*p.i.*). Afterwards an axially oriented T1w FLASH sequence will be sampled in two stacks placed over epi- and mid-gastrium and over the pelvis. In case of suspected perineal/perirectal involvement further high-resolution sequences will be acquired in additional sagittal orientation, optionally with fat suppression (Table 2).

DISEASE EVALUATION USING MRI

MR imaging can detect a number of intestinal and extraintestinal findings which should be included in the report (Table 3). First of all the intestinal distension and caliber changes have to be described. Afterwards intestinal and mesentery pathologies have to be mentioned.

Transmural abnormalities, wall thickening, loss of layering, cobblestoning, ulceration, pseudo polyps, mural abscess, pseudo sacculation appearance, stenosis, strictures, prestenotic dilation can be described without contrast application by assessing high resolution T2w sequences.

Thickening and pronounced enhancement of the intestinal wall

Bowel wall thickness of more than 3 mm is considered pathologic[3]. Swelling of the wall is a result of interstitial edema. Increased signal in the bowel wall in edema sensitive T2w images (*e.g.*, sequence-short tau inversion recovery, STIR) may be suggestive for active disease (Figure 5). Hyperemia results in more or less increased signal intensity on T1w sequences after Gadolinium application, depending on disease activity (Figure 6). The degree of

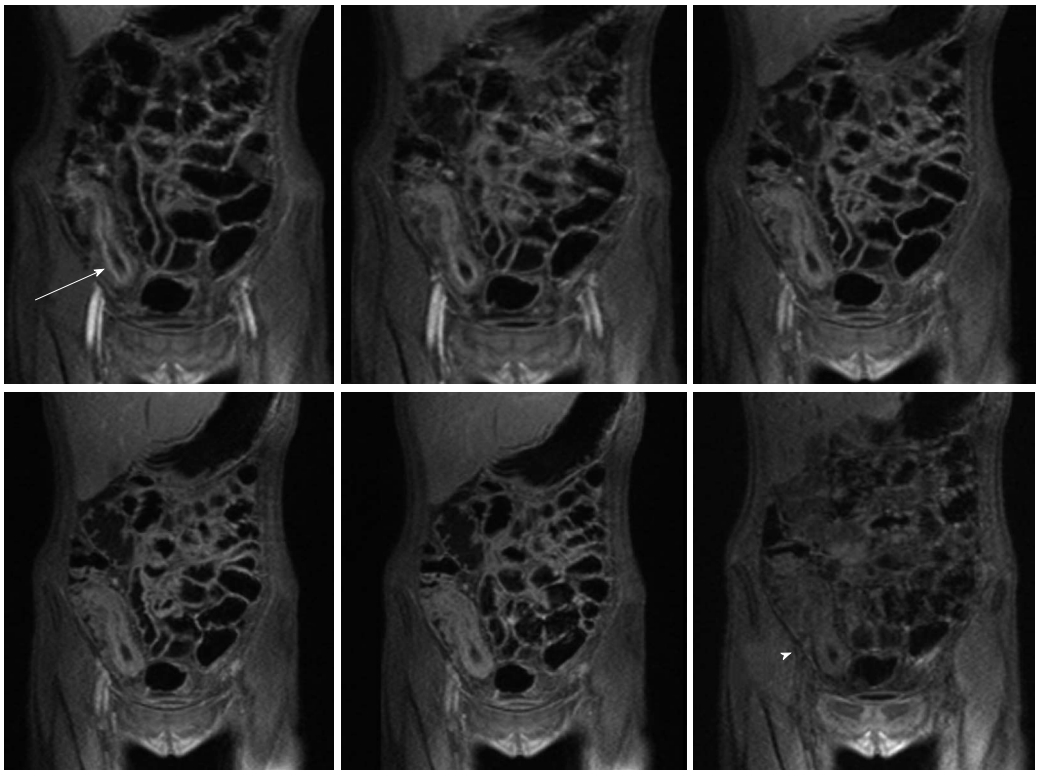

Figure 4 Dynamic contrast enhanced T1-volume interpolated gradient-echo sequence demonstrating different phases of signal increase in the various layers of the bowel wall [at first mucosa (arrow), followed by the serosa, the muscularis, and finally the submucosa (arrowhead)].

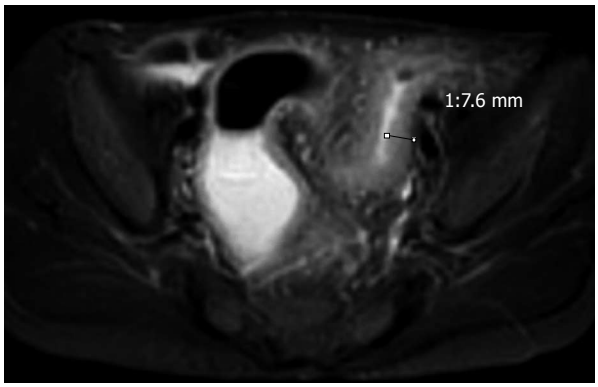

Figure 5 Eleven year old boy with Crohn's disease. T2 weighted sequence with fat saturation (Tirm/Stir) demonstrates thickening of the bowel wall and hyperintense signal corresponding to edema in acute inflammation. Narrowing of the lumen.

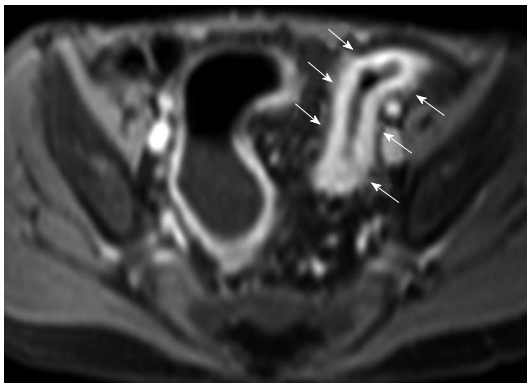

Figure 6 Eleven year old boy with Crohn's disease. T1 weighted contrast enhanced sequence shows strong transmural enhancement (arrows).

bowel enhancement following contrast application (0.1 mmol/kg body weight Gd DTPA *iv*) by evaluation the increase in signal intensity (SI) can help to differentiate active from chronic disease[29]. For quantification, Alexopoulou defined the percentage contrast enhancement as %CE = [(SI bowel postcontrast - SI bowel precontrast)/SI bowel precontrast] × 100. A layered enhancement pattern on T1w scans can be highly specific[29]. The use of contrast-enhanced MRI (CE-MRI) may be useful in diagnosing IBD, as well as in the differentiation between CD and UC[32]. A study in children by Laghi *et al*[33] correlated the semiquantitative score findings of MRI with endoscopic, histological, and CD activity index findings. MR showed a sensitivity of 84% and a specificity of 100%. Other studies showed no correlation between percental contrast enhancement of the bowel wall and the CD activity index in children[29]. New techniques like diffusion weighted imaging (DWI) are helpful adjuncts. DWI can be performed as transverse free-breathing echo planar imaging (EPI) sequence with diffusion-sensitizing gradients applied sequentially along the three orthogonal directions[34,35]. Using a diffusion gradient of three different b values (0, 50, 800 s/mm^2) the sensitivity and specificity compared to surgery and/or conventional enteroclysma was 86% and 97% in adults suffering from CD[34]. Neubauer *et al*[36] could prove DWI in combination with high-resolution T2w-HASTE is equal (if not superior) to CE-

Table 2 Sequence parameters used for magnetic resonance enterography (magnetic resonance avanto, magnetic resonance symphony)

Sequence	Orientation	Slice thickness (mm)	Time of repetition (ms)	Time of echo (ms)	Time of inversion (ms)	Flip angle (°)
TRUFI	Coronal	6	3.8	1.9		80
T2	Coronal	70-100	3000	985		180
TRUFI	Transverse	4	4.3	2.15		80
HASTE	Transverse	4-7	1000	87		150
VIBE	Coronal	2	3.18	1.1		9
FLASH	Transverse	4-5.5	233	1.91		70
TIRM	Transverse	8	5050	91	140	180
DWI	Transverse	5	5500	139		0

TRUE: True steady-state free precession images; HASTE: Half-fourier acquisition single-shot turbo-spin-echo; FLASH: Fast low-angle shot T1w sequences; VIBE: Volume interpolated gradient-echo; DWI: Diffusion weighted imaging.

Table 3 Magnetic resonance imaging features in inflammatory bowel disease in children differentiation active and fibrostenotic phase

Imaging features	Active inflammation	Fibrostenotic disease
Mural thickening	Moderate	Mild
Mural enhancement	Avid	Mild
Stratified enhancement	Yes	Variable
Mural edema	Yes	Mild/absent
Mesenteric adenopathy	Yes	No
Fibrofatty proliferation	Variable	Yes
Abscess, empyema, fistula	Complicated disease	Variable

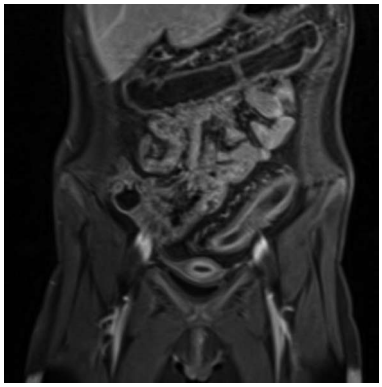

Figure 7 Stratified enhancement in the sigmoid in a female suffering from ulcerative colitis.

MRI for detecting inflammatory lesions in children with CD. Based on these two sequences imaging without the need of contrast media seems to be sufficient for diagnosis, reducing scanning time to less than 10 min. Using a high *b* value of 800 s/mm^2 for DWI the background signal arising from non-inflamed tissue and from body fluids can be largely suppressed, so that inflamed bowel segments are more easily detected. The same authors observed a reduction of the apparent diffusion coefficient (ADC) in the inflamed bowel segments based by an altered diffusivity of extracellular water in inflamed bowel wall tissue. This could also be shown by another study in children with terminal ileitis using a *b* value of 500 s/mm^2[37]. In this study there was a significant correlation between the bowel wall minimum ADC and established MRI markers of disease activity (bowel wall thickening, striated pattern of arterial enhancement, degree of arterial enhancement, degree of delayed enhancement, amount of mesenteric inflammatory changes, and presence of a stricture).

Mural stratification

The inflammatory process in CD results in a complete loss of the intestinal layering which can be detected by high-resolution MRI. The transmural aspect of CD results in homogeneous high signal intensity of the entire bowel wall without normal stratification after administration of Gd. Exclusive involvement of the mucosa is a typical sign of UC. The result is a stratified enhancement the so-called target or double halo appearance (Figure 7). So, in the examination report the two patterns of enhancement can be described as "homogeneous" or "stratified"[22]. The stratified enhancement pattern is typical for long-standing CD with fibrosis or in patients following intensive treatment.

Cobblestones and ulcers

Cobblestone formation is typically found in UC patients and less often in CD. The development of aphthous lesions along the mucosa result in deep linear or stellate lesions. Deep ulcers can be found in CD patients.

Strictures and stenosis

Whenever there is a procedural adequate distension of the bowel a reduction of the diameter (normally around 2.5 cm) is suspected a stricture. Chronic IBD-affected bowel segments may result in strictures. The presence of a pre-stenotic dilation may help in the diagnosis. Long-standing fibrotic strictures with a thick hypointense wall can be diagnosed on heavily T2w sequences. Notice that there is no significant contrast enhancement. Inflamed bowel segments tend to show a reduction of the size of the intraluminal diameter. The strictures can be demonstrated more sensitively using dynamic filling of the bowel with MR enteroclysma technique compared to hydro-MRI technique (Figure 8). In the case of lymph node bulking the bowel diameter can also be reduced. Description of localization and length of the involved bowel segment is necessary for planning surgery.

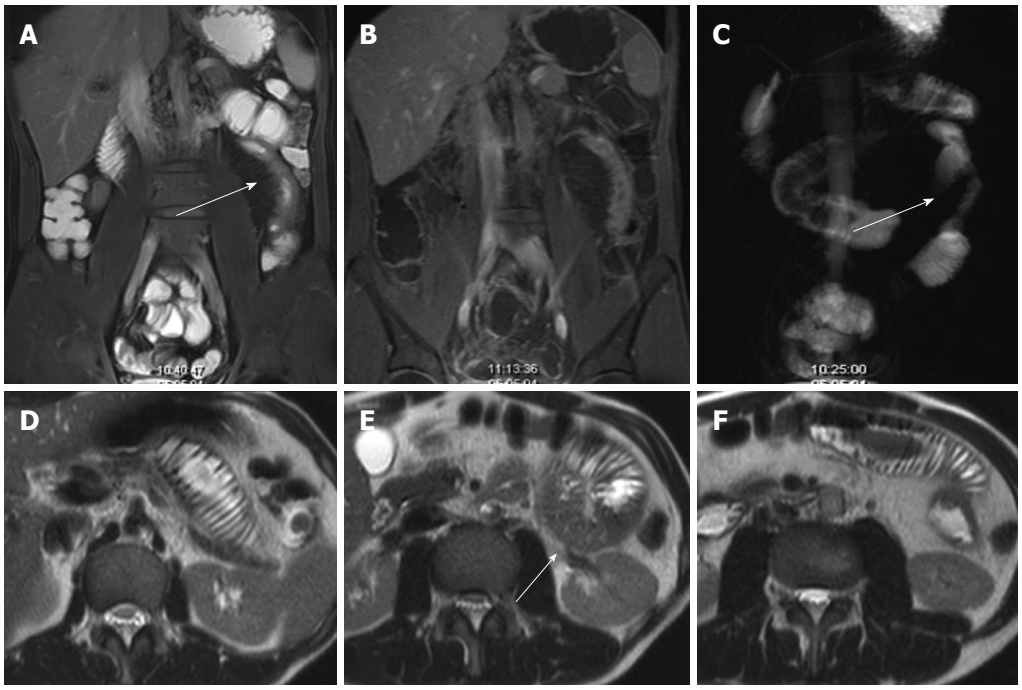

Figure 8 Stricture in inflammatory bowel diseases-chronic phase, no activity of inflammation. There is no edema with reduced signal intensity in T2 weighted sequences (A, D, arrow). The MR enteroclysma does not show any widening of the involved jejuna bowel loop (C, arrow), also seen in axial T2w sequence (D-F). There is moderate enhancement (B).

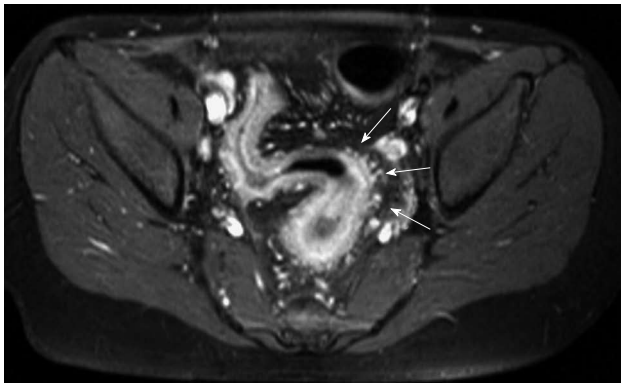

Figure 9 Twelve year old female with Crohn's disease. Comb sign. CE-T1 weighted sequence with fat saturation. Blood vessels within the mesentery (arrows).

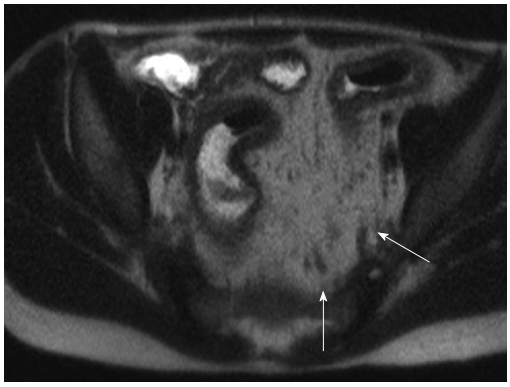

Figure 10 Eleven year old male with Crohn's disease. Creeping fat sign (arrows).

Extramural findings

The extramural signs of IBD such as free fluid, peritoneal fat stranding and enhancement, mesenteric edema, fibrofatty proliferation, the comb sign, mesenteric lymph nodes, abscesses, fistulas can be easily detected by MRI. Especially for the evaluation of perineal complications MRI is the gold standard nowadays. In a meta-analysis including 33 articles. Horsthuis *et al*[38] showed the highest sensitivity in MR enteroclysma compared to hydro MRI, however, in children sensitivity was lower than in adults. One reason may be the lower amount of mesenteric fat which helps in separating the bowel loop segments for image interpretation. Motion artifacts, reduced complicance might be further possible reasons.

Comb sign

This is a sign of active inflammation (typical of CD) describing the vascular engorgement of the vasa recta on the mesenteric side of the bowel wall. The comb sign may be appreciated well on fat-saturated sequences as multiple tubular, sometimes tortuous vessels, aligned like the teeth of a comb (Figure 9).

Creeping fat sign

The chronic inflammation of the mesenteric fatty tissue induces a proliferation of the fat tissue itself together with a fibrotic component along the mesenteric border of inflamed bowel segments. This is the so called fibro-fatty proliferation which is also a typical CD sign. MR imaging can show a pseudo-mass which is surrounding the bowel

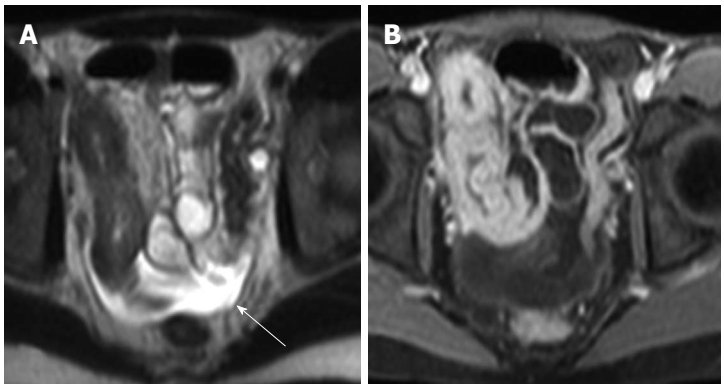

Figure 11 Thirteen years old girl with Crohn's disease. A: T2w image demonstrating strong hyperintense ascites. Arrow marks the free ascites; B: T1w sequence after contrast application. Fluid is not as easily detected as in T2w images. T1w: T1 weighted; T2w: T2 weighted.

Figure 12 Coronally oriented true steady-state free precession images shows enlarged mesenteric lymph nodes (arrows).

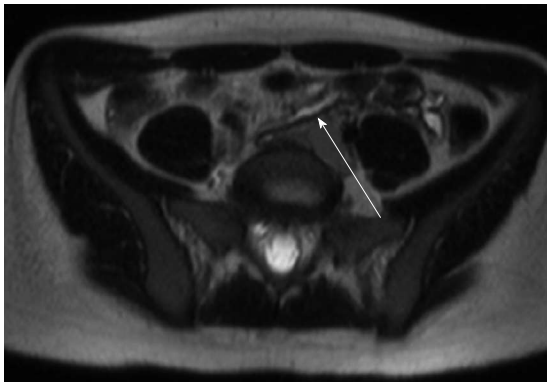

Figure 13 Seventeen years female with Crohn's disease. T2 weighted sequence showing enterocolic fistula (arrow).

loop with intermediate T2w signal intensity corresponding to fibrous or/and fatty components (Figure 10).

Free fluid

The distribution and amount should be described. Using the signal intensity on T1w and T2w sequences different entities (blood, pus, ascites) can be suspected (Figure 11). Diffusion weighted imaging is helpful in distinguishing serous fluid and empyema/pus.

Mesenteric lymph nodes

Mesenteric lymphadenopathy is well-depicted using TRUFI or T2w TSE sequences (Figure 12). In case there are multiple and round lymph nodes larger than 10 mm in diameter lymphoma needs to be excluded. As first choice follow-up imaging using ultrasound is recommended in these patients.

Fistula, abscess, phlegmon

Fistulas can be categorized as enteroenteric, enterocolic, enterovesical, enterocutaneous, or complex perianal (Figure 13). Fistulas are typically found in sites where two inflamed bowel segments are in close proximity to each other or in regions with high-grade bowel stenosis[39]. Typically, the fistulous tract shows strong enhancement which can be differentiated using fat-suppressed T1w high-resolution sequences. The direct visualization of the fistulous tract is not always possible, but indirect signs can be recognized. So, around the sinus tract inhomogeneity and enhancement of the mesenteric fat can be demonstrated. Other extraintestinal findings may be a psoas muscle abscess, and sacroiliitis (Figure 14). In these cases MRI of the pelvis will be recommended.

LIMITATIONS AND DEVELOPMENTS

The greatest limitation of MR enterography especially in children is the dependence on a compliant patient. It should be considered, that the patient is strained by fluid placement, application of contrast agents and motility influencing agents, and breath-holding maneuvers. Artifacts may be the result of bowel, breathing, and patient moving. In order to reduce these motion artifacts, two techniques can be applied: parallel imaging, navigator techniques, and motion correction[40]. The use of higher field strength results in a greater signal-to-noise-ratio and has the potential of reducing scan time and increasing the spatial resolution[5,28]. However, there is a concurrent increase in artifacts, especially chemical shift artifacts and susceptibility artifacts in abdominal imaging using gradient-echo imaging like TRUFI, CE-T1 VIBE, and FLASH. The T2w HASTE is more robust[41]. Con-

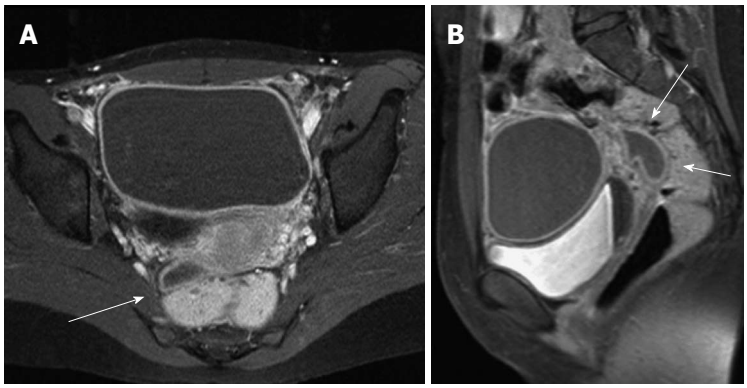

Figure 14 Female with Crohn's disease. Sagittal T1 weighted sequence allows to describe presacral abscess (arrow). Fat saturation was applied. A: Axial image; B: Sagittal image (arrows marking abscess; late contrast phase. Notice sedimentation of different components in the bladder).

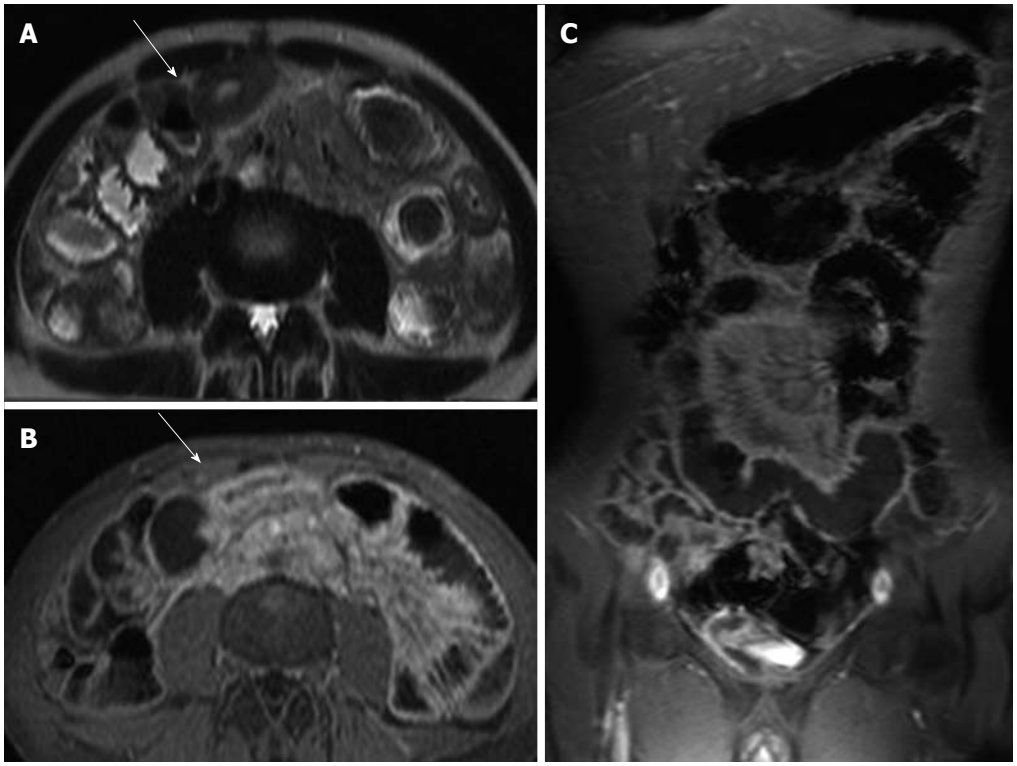

Figure 15 Magnetic resonance enterography allows an overview about involved bowel segments. In this case there was an isolated involvement of the jejunum. A: Axial T2w sequence showing thickened bowel wall without edema; B, C: Axial and coronal fat saturated contrast-enhanced T1w sequence demonstrating transmural enhancement in Crohn's disease. T1w: T1 weighted; T2w: T2 weighted.

sequently, the feasibility of high-field MRI in children suffering from IBD should be studied in the future. The MR study is time consuming and staff-intensive. So, the use of a modified examination consisting of Diffusion weighted imaging with high *b* values from 600 to 1300 s/mm^2[5] and high resolution T2w sequences (isovoxel 3D with multiplanar reformation in three orthogonal directions) has to be evaluated in the future as a standard examination protocol resulting in a scanning time of not more than 10 min. This protocol can be used for routine controls. But, in the case of newly diagnosed IBD and in cases of relapse the examination has to be more extensive.

CONCLUSION

There are still sites that prefer fluoroscopic contrast studies and multislice CT in the diagnosis of IBD, also in children. But, the development of the last decade promises the increasing value of MR imaging in the evaluation of the intestine. MR enterography has definitely advantages including the detection and assessment of disease activity of the entire gut and the ability to evaluate extraluminal disease (Figure 15) and is therefore becoming the standard assessment of the small bowel in many centers. The radiation free ultrasound performed with adequate technique and experience by the sonographer should

be used as initial imaging method. MR imaging should be performed at the initial diagnosis of CD and should considered in any case of treatment changes, especially if surgery is planned.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Role of the gut microbiota in inflammatory bowel disease pathogenesis: What have we learnt in the past 10 years?

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Abstract

Our understanding of the microbial involvement in inflammatory bowel disease (IBD) pathogenesis has increased exponentially over the past decade. The development of newer molecular tools for the global assessment of the gut microbiome and the identification of nucleotide-binding oligomerization domain-containing protein 2 in 2001 and other susceptibility genes for Crohn's disease in particular has led to better understanding of the aetiopathogenesis of IBD. The microbial studies have elaborated the normal composition of the gut microbiome and its perturbations in the setting of IBD. This altered microbiome or "dysbiosis" is a key player in the protracted course of inflammation in IBD. Numerous genome-wide association studies have identified further genes involved in gastrointestinal innate immunity (including polymorphisms in genes involved in autophagy: *ATG16L1* and *IGRM*), which have helped elucidate the relationship of the local innate immunity with the adjacent luminal bacteria. These developments have also spurred the search for specific pathogens which may have a role in the metamorphosis of the gut microbiome from a symbiotic entity to a putative pathogenic one. Here we review advances in our understanding of microbial involvement in IBD pathogenesis over the past 10 years and offer insight into how this will shape our therapeutic management of the disease in the coming years.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Gut microbiota; Innate immune response; Probiotics; Prebiotics; Faecal transplant

Core tip: In the last decade there have been enormous strides in our understanding of the role of gut microbiota in the aetiopathogenesis of inflammatory bowel disease (IBD). Newer molecular and genetic diagnostic tools have elucidated distinct changes in the gut microbiota in IBD patients and clarified the deficiencies of innate immunity. A link between environmental factors like diet, host immunity and the gut microbiota has been established. This review aims to enumerate these diverse strands of converging research in the last decade to outline the exciting prospects of possible personalized therapeutic interventions for patients with IBD in the coming years.

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises two distinct

conditions, ulcerative colitis (UC) and Crohn's disease (CD) that are characterized by chronic relapsing inflammation of the gut in genetically susceptible individuals exposed to defined environmental risk factors[1,2]. IBD was historically considered to be a "Western" disease but in the last decade there has been a definite increase in its incidence and prevalence suggesting that it is progressively emerging as a global epidemic[3]. In the high prevalence regions the incidence of IBD has continued to rise in the past decade[4,5].

There has been a parallel rise in our understanding of the critical role of the gut microbiota in the aetiopathogenesis of IBD. This is aptly exemplified by entering the key words, "microbiota" or "microflora" and "inflammatory bowel disease" into the PubMed database. On restricting the search to the last 10 years, over 800 articles published on this subject can be retrieved as opposed to 100 articles in the decade preceding it. This radical explosion of interest has been primarily due to the advent of culture-independent techniques like next generation sequencing and metagenomics which has enabled the global assessment of the gut microbiota much more accurately and in a vastly more sophisticated manner[6,7]. The largest and perhaps the most ambitious initiative that has emerged in the last decade is the NIH sponsored Human Microbiome Project with a total budget of $115 million to study the changes of the human microbiome in health and disease[8]. It has recently led to the publication of 5177 microbial taxonomic profiles from a population of 242 healthy adults sampled at 15 or 18 body sites up to three times, with over 3.5 terabases of metagenomic sequence so far, which will serve as a comprehensive framework for future research in this field[9].

This expansion of knowledge in the last decade has also shifted the search from external environmental triggers to a trigger within the complex luminal microbiome or the so called "in-vironment" that we harvest within ourselves[10-12]. Prior to these radical developments research had focussed on unearthing a pathogen amidst the vast plethora of microbes in the gut lumen, which could be held responsible for initiating the inflammatory cascade that is typical of IBD[13]. This endeavour was akin to searching for the veritable "needle in the haystack". The findings in the last decade has turned this whole concept on its head by revealing that the gut microbiome as a whole is altered in IBD, suggesting that perhaps the entire "haystack" is faulty. This concept of an altered gut microbiome or dysbiosis is possibly the most significant development in the field of IBD research in the past decade.

The other major shift in our knowledge of the aetiopathogenesis of inflammatory bowel disease has been from the host perspective. The dogma that CD and UC are typical autoimmune disorders was based on the characteristic histological appearance of these conditions and the response to immune-modulator drugs but the veil has lifted from this deep-embedded misconception[14,15]. Over the past decade, genome wide association studies and newer genetic technologies have elucidated distinct genetic defects in IBD patients. This has particular relevance with respect to host-microbial interaction at the luminal surface in the gut. A similar analysis on the PubMed database with the search items "genetics" and "inflammatory bowel disease" leads to a staggering yield of more than 5600 publications in the last decade as opposed to 2000 articles in the decade prior. It must be said that the avenue of research in this field was first opened up in 2001 when the first association of the nucleotide-binding oligomerization domain-containing protein 2 (NOD-2) gene mutation and susceptibility to Crohn's disease was documented[16,17]. This has resulted in a drastic paradigm shift wherein IBD is no longer considered an autoimmune disease but may be an immunodeficient condition instead[15]. This putative genetic susceptibility leads to a complex interaction between the diverse gut microbiome and the local innate immune system and forms the current basis for the aetiopathogenesis of IBD (Figure 1).

DYSBIOSIS

The normal gut microbiome comprises 100 trillion diverse microbes, mostly bacteria, encompassing over 1100 prevalent species, with at least 160 species in each individual[18]. An exhaustive analysis of normal global gut bacterial communities suggests the possible existence of distinct enterotypes (*Bacteroides*, *Prevotella* or *Ruminococcus*) which are predominantly driven by dietary intake but independent of age or BMI[19,20]. Further analysis suggests that the *Bacteroides* enterotype is associated with a "western" protein rich diet as opposed to the *Prevotella* enterotype which was associated with a carbohydrate rich diet[21]. It remains to be seen whether this western enterotype turns out to be a distinct risk factor for developing IBD.

Dysbiosis or a definitive change of the normal gut microbiome with a breakdown of host- microbial mutualism is probably the defining event in the development of IBD. The shift from predominant "symbiont" microbes to potential harmful "pathobiont" microbes has now been well documented[22]. Some of these changes in the gut microbiome have been detected in the common subset of IBD patients but some have been clearly delineated either in CD or in UC patients. The most well defined change that has been noted in patients with IBD is the reduced abundance of the phyla *Firmicutes*[23-25]. Amongst the *Firmicutes*, the reduced presence of *Faecalibacterium prausnitzii* has been well documented in patients with CD as opposed to controls[23,26-30]. This has been countered in a paediatric cohort of patients with CD where there were increased levels of *Faecalibacterium prausnitzii* suggesting a more dynamic role for this bacterium with a putative protective effect at the point of onset of IBD[31]. In addition, there was a definite decrease in diversity of *Firmicutes,* with fewer of its constituent species detected in patients with IBD[23,32,33]. Unlike *Firmicutes*, there have been reports of increased number of bacteria from the phylum *Bacteroidetes* in patients with IBD[34-36]. Paradoxically, there

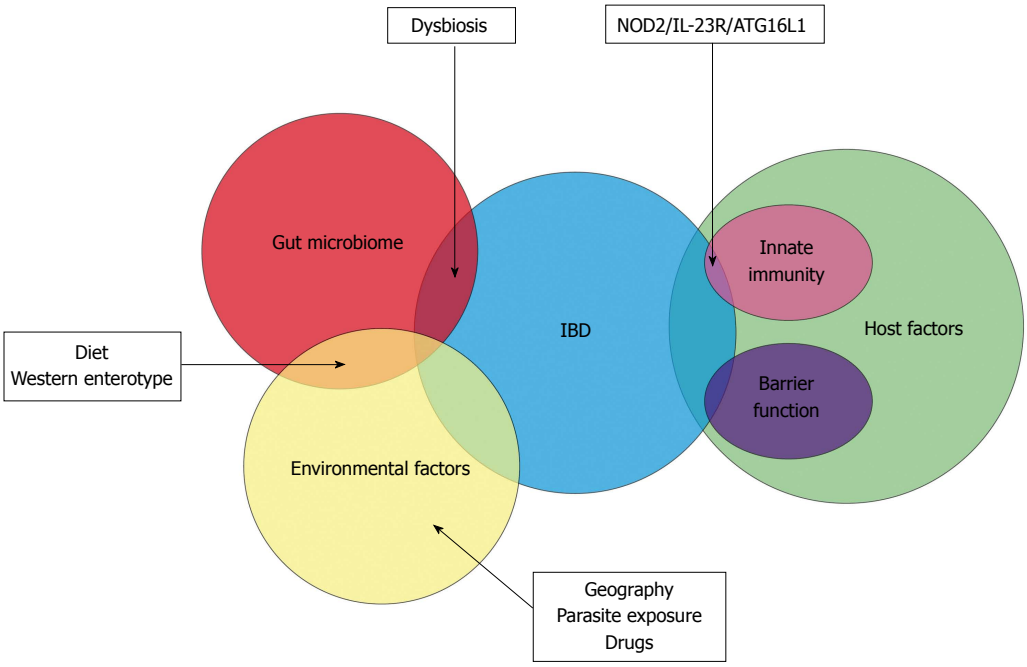

Figure 1 The Venn diagram depicts the overlapping role of the gut microbiome, host and environmental factors in the aetiopathogenesis of inflammatory bowel disease. Dysbiotic changes in the gut microbiome may be influenced by diet and other environmental factors and predispose to inflammatory bowel disease (IBD). A small proportion of IBD patients have demonstrable genetic susceptibility factors. NOD2: Nucleotide-binding oligomerization domain-containing protein 2; ATG16L1: Autophagy related protein 16-like 1; IL-23R: Interleukin 23 receptor.

have been some studies which have shown reduction in these bacterial species as well[23]. There is a suggestion that there may be spatial reorganization of the *Bacteroides* species in patients with IBD, with *Bacteroides fragilis* being responsible for a greater proportion of the biofilm mass in patients with IBD compared to controls, suggesting increased adherence[37]. Bacteria belonging to these two phyla make up for 90% of the phylogenetic categories in the normal microbiome and it is interesting to see the disparate ways in which they are altered in IBD.

Most of the known pathogenic bacteria in humans belong to the phylum *Proteobacteria*, which have been increasingly found to have a key role in IBD[38]. Microbial diversity analysis has shown a shift towards an increase in bacterial species belonging to this phylum, suggesting an aggressor role in the initiation of chronic inflammation in patients with IBD[39-42]. More specifically, increased concentrations of *Escherichia coli* including pathogenic variants have been documented in ileal CD[28,43].

This interesting shift within the gut microbiome with a decrease in obligate anaerobes of the phylum *Firmicutes* and an increase in facultative anaerobes of *Proteobacteria* has given rise to a putative "oxygen" hypothesis wherein disruption in anaerobiosis points to a role for oxygen in intestinal dysbiosis[44]. Similar functional disruptions associated with changes of the gut microbiome in patients with IBD may have more long reaching effects. Metagenomic analysis has revealed that the altered microbiome in IBD has 25% fewer genes and metaproteomic studies have shown a depletion of proteins and functional pathways[18,45]. The ileal CD patients were found to have alterations in bacterial carbohydrate metabolism, bacterial-host interactions, as well as human host-secreted enzymes[45]. Elucidation of the functional impact of the changes seen as a result of dysbiosis will help design remedial measures that will help in the treatment of IBD patients.

The immediate question which follows is how the host responds to dysbiosis. Host genetics factors, specifically those pertaining to the innate immunity arm, is expected to play a role in the aetiopathogenesis of IBD. The "chicken and the egg" question is what comes first. Are the changes in the gut microbiome a result of an aberrant immune response in a genetically susceptible individual or does the abnormality in the gut microbiota lead to an aberrant immune response in such an individual? Twin studies have shown that disease phenotype rather than host genotype plays a greater role in determining changes in gut microbiota[46]. However, studying the microbiota in subsets of patients with and without NOD2 and autophagy related protein 16-like 1 (ATG16L1) risk alleles showed that the affected genotypes were significantly associated with microbial compositional change but disease phenotype played a role as well[47]. The confounding factor is that these two alleles are associated with ileal CD and not colonic CD. It makes it difficult to attribute these genetic defects as a cause of dysbiosis but highlights the intricate role of innate immunity in IBD.

INNATE IMMUNITY AND IBD

The gastrointestinal microbiota is a major source of immune stimulation. The colonic epithelium lies in close proximity to a high density of diverse microbes leading to a continuous network of communication between

host cells and microbes. This continual communication is essential for the maintenance of normal homeostasis though contribution to processes including supply of nutrients, xenobiotic metabolism and protection from pathogenic microorganisms, can have deleterious effects and contribute to intestinal inflammation[48,49]. In patients with IBD this delicate balance is disturbed as a result of host immune defects in microbial recognition or handling/clearance strategies[50]. Pattern recognition receptors (PRRs) are essential in distinguishing "friend from foe" in this very complex interaction and hold the key to understanding how genetic factors lead to an abnormal immune environment wherein normal commensal organisms can lead to pathological chronic inflammation. Ten years ago toll-like receptors (TLRs) and NOD2 were known to be involved in IBD pathogenesis although our understanding of their location, function and involvement was still very rudimentary. Evidence from IBD genetic studies had demonstrated that several innate immune genes had functionally relevant polymorphisms. Of those studied NOD2 genetic variants confer the greatest risk.

A decade ago the novel association between the recently characterised TLR4 Asp299Gly was described for both CD and UC[51]. This finding supported previous evidence of PRR genetic influence in IBD susceptibility which had shown that polymorphisms in NOD2 (Arg-702Trp, Gly908Arg, and leu1007fsinsC) and the CD14-159C/T promoter polymorphism were associated with CD[16,17]. Since then, additional polymorphisms in TLRs have been identified including TLR1 R80T and TLR2 R753G which have been associated with pancolitis in UC patients[52]. The TLR9-1237T/C promoter polymorphism (TLR9-1237), which is associated with increased nuclear factor kappa B (NF-κB) binding affinity, has also been associated with CD[53,54].

The normal colonic epithelium constitutively expresses a variety of PRRs although expression levels are generally low with many receptors located basolaterally thus preventing interaction with luminal antigens[55]. Nevertheless, intestinal epithelial cells are responsive to TLR ligands and recognise/respond to commensal bacteria secreting antimicrobial proteins and cytokines which facilitate intercellular interactions[48]. Primary human intestinal epithelial cells express constitutive TLR3 and TLR5 and low levels of TLR2 and TLR4[56]. TLR2, 4 and 5 are expressed on the cell surface and recognise extracellular microbes. TLR3 detects viral particles and is located intracellularly on early endosomal vesicles. The critical role of TLR4 as a first line of defence against potential bacterial pathogens is now beyond doubt. Impairment of TLR4 function permits bacterial invasion and persistence, and leads to the characteristic inflammation of IBD. The importance of TLR5 in intestinal homeostasis has also been effectively demonstrated using microbiota transfer from knockout mice[57,58].

Distinct changes in TLR expression have been documented in IBD. TLR4 is found to be up regulated in both UC and CD, whereas the levels of TLR2 and TLR5 remain unchanged[56,59]. Altered TLR2 and TLR4 expression has been documented in the intestinal macrophages compared to peripheral monocytes, and a higher percentage of intestinal dendritic cells (DCs) have been shown to express TLR2 and TLR4 in IBD compared to control subjects[59]. Intestinal macrophage signalling through PRRs has also been shown to be affected by increased expression of suppressor of cytokine signalling 1 and sterile and Armadillo motif-containing protein[60,61]. Dysregulation of β-catenin and phosphotidylinositol-3-kinase pathways are also involved with alterations in these pathways involved in colitis susceptibility[62,63]. In IBD patients increased cytokine production is seen by lamina propria DCs and macrophages, consistent with dysregulated tolerance[64-66]. These changes can explain some of the abnormal response to the resident gut microbiota. However, it is difficult to elucidate whether the change in TLR expression initiates disease or is an epiphenomenon resulting from pro-inflammatory cytokine release. In many cases absence of epithelial cell-derived antimicrobial pathways increases susceptibility to intestinal inflammation, with IBD patients expressing lower levels of α-defensin compared to healthy individuals[67,68].

Animal models had played a significant role in driving forward our understanding of IBD pathogenesis, especially murine colitis models. TLR4 and MyD88 knockout mice have been shown to demonstrate distinctly less pathology following chemical induction of colitis with dextran-sodium sulphate although bacterial translocation to mesenteric lymph nodes was more commonly detected[69]. Impairment of TLR4 and TLR5 function has been shown to facilitate bacterial invasion and persistence (TLR4) and impact on intestinal homeostasis; development of metabolic syndrome (TLR5)[57,58].

Alterations in NOD2 function due to genetic polymorphisms have demonstrated an inability to respond to bacterial muramyl dipeptide (MDP) leading to ineffective downstream signalling of NF-κB[70]. NOD2, is expressed on several different cell types including myeloid-derived, epithelial and endothelial cells. As with impairment of TLR function, NOD2 deficiency increases translocation of enteric bacteria to the lamina propria, with alterations in cytokine expression following exposure of peripheral blood mononuclear cells to MDP also reported potentially explaining the alterations in cytokine profiles typically seen in CD[71,72]. Interestingly, NOD2 has more recently been shown to respond to viruses[73]. With increasing interest in non-bacterial microbes in IBD pathogenesis, namely viruses and fungi, this may prove to be an increasing area of consideration. A decrease in the protective, anti-inflammatory Th-2 cytokine IL-10 has been documented in NOD2 mutants further adding to our understanding of the functional abnormalities characteristic of CD[74].

Counter-intuitively, NOD2 can also contribute to down-regulation of inflammatory responses with chronic stimulation of NOD2 acting to tolerise cells against bac-

terial stimulation and ultimately down regulating other PRRs[75-77]. Hence, in CD patients with dysfunctional NOD2 this restraint is removed and the inflammatory response from other PRRs increases.

NOD2 is also implicated in mechanisms of microbial killing. Autophagy, an important mechanism of microbial cell clearance, is regulated through PRRs[78]. NOD2 interacts with ATG16L1[78,79]. Therefore dysregulation of NOD2 impacts not only on microbial recognition but also handling. Genetic variants in ATG16L1 and also a second autophagy gene, immunity-related GTPase family M have been associated with CD[80,81].

ROLE OF INDIVIDUAL PATHOGENS IN IBD

The rapid development of molecular techniques has also kindled hopes in the search for specific pathogenic agents initiating the inflammatory process of IBD. The pathophysiology of IBD does suggest that either primary or secondary pathogens play an important role in the cycle of inflammation. Many organisms have been proposed but those deemed to have been of the most interest over the last ten years are discussed below (Table 1).

Mycobacterium avium subspecies paratuberculosis

Mycobacterial infection has been postulated in the aetiology of Crohn's disease since its first description in 1913. The association stems from the observed similarity between Crohn's disease and the bovine condition Johne's disease, a condition caused by *Mycobacterium avium paratuberculosis* (MAP) infection leading to granulomatous enterocolitis. There have been vast numbers of studies in this area but the role of MAP remains uncertain[50,82,83].

MAP can be widely isolated from meat, dairy products and water, indicating sources of infection and supporting its role[82,83]. However, a large study found a lack of epidemiological support for environmental exposure[84]. Over the last decade research into the prevalence of MAP in IBD patients has been inconclusive. A large number of researchers have successfully shown a higher prevalence of MAP in Crohn's patients compared to controls but, it seems for each of these there has been an equivalent study yielding no association[85-97].

In support of its role the ability of MAP to invade gut epithelial cells, inducing tissue damage and inflammation, has been shown[98]. A dominant T-cell response to MAP has also been seen in CD patients and macrophages infected with viable MAP are associated with high production of tumour necrosis factor-alpha (TNF-α), a marker for CD[95,99,100]. Using mouse models, MAP has been found to induce full-thickness necrotizing colitis after subcutaneous and transluminal injection[101].

The discovered association between CD and the autophagy gene *ATG16L1* lends further credence to the theory as it is known that autophagy is essential for inhibition of mycobacterium tuberculosis in infected macrophages[102,103]. Defective innate immune killing mechanisms in patients with *NOD2* polymorphisms at first also seem to support the idea, and indeed it has been found that monocytes heterozygous for a *NOD2* polymorphism are more permissive to the growth of MAP[104]. Beyond contemplation, however, evidence for this hypothesis is limited. MAP has been detected most commonly in colonic disease; this is in direct contrast with the prevalence of *NOD2* mutation in ileal disease[105,106]. In fact a study directly looking at the relationship between NOD-2 and MAP serology found no association[107]. Combining this with the response of CD to immunosuppressant and anti-TNF therapy, known to cause MAP proliferation and a lack of success of anti-mycobacterial therapy, the role of MAP is clearly still in doubt[108].

Helicobacter

Helicobacters, as human gastrointestinal pathogens, have assumed great research interest since the discovery by Robin Warren and Barry Marshall of *Helicobacter pylori* as the infectious agent in gastric and duodenal ulceration. Also, similar to MAP, one of the main prompters of research into the role of *Helicobacter* in IBD has been their propensity to cause colitis in animal models like Cotton-top tamarin monkeys (*Saguinus oedipus*). Despite this, there has been a lack of success in the last decade establishing presence of *Helicobacter* in IBD patients. The findings of studies looking into the molecular evidence of *Helicobacter* presence are varied and studies aimed at culturing viable *Helicobacter* from IBD tissue have failed[109-121]. Interestingly the only seemingly universally accepted action of *Helicobacter* in IBD is the apparent protective effect of *Helicobacter pylori* which has convincingly been found to be negatively correlated with IBD[113,118-122]. This may conform to the "hygiene hypothesis" for the development of IBD[123].

The evidence for an association is much stronger with enterohepatic *Helicobacter* species. Non-*pylori Helicobacter* organisms have been shown to induce colitis in a number of rodent models; *Helicobacter hepaticus* and *Helicobacter bilis* (*H. bilis*) most prominently but, also *Helicobacter trogontum*, *Helicobacter rodentium* and *Helicobacter typhlonius* with cytokine patterns which were very similar to that of human IBD[124-128]. When studying the response to *H. bilis*, Jergens *et al*[126] showed that there was an IgG mediated response to the microbiota prior to the development of colitis, suggesting the ability of *H. bilis* to induce the hosts immune response to commensal bacteria, leading to the observed immune-mediated intestinal inflammation of IBD. In human subjects, enterohepatic *Helicobacter* species prevalence was significantly higher in colonic biopsy samples from patients with UC group compared to control subjects[118].

Campylobacter

Campylobacter is a relatively new and important player in IBD. Unlike the other IBD suspects, *Campylobacter* does not have a suitable animal disease model; instead interest stems from the recognition of *Campylobacter jejuni* (*C. je-*

Table 1 Evidence for the role of specific major pathogens in the aetiopathogenesis of inflammatory bowel disease in the last decade *n* (%)

Year	Pathogen	Disease	Sample type	Detection rate			Reference
				CD	UC	Control	
2003	MAP	CD	Tissue	34/37 (92)		9/34 (26)	[85]
2003	MAP	CD and UC	Tissue	0/24 (0)	1/28 (4)	6/19 (32)	[94]
2003	*Helicobacter*	CD and UC	Tissue	0/9 (0)	0/11 (0)	0/10 (0)	[109]
2004	MAP	CD and UC	Blood	107/283 (37.8)	50/144 (34.7)	135/402 (33.6)	[92]
2004	MAP	CD and UC	Blood	13/28 (46)	4/9 (45)	3/15 (20)	[86]
2004	*H. pylori*	UC	Tissue	8/42 (19)		7/74 (9.5)	[110]
2004	*Helicobacter*	CD and UC	Tissue	1/25 (4)	5/33 (15.2)	0/29 (0)	[111]
2004	*Helicobacter*	CD and UC	Tissue	0/30 (0)	0/26 (0)	0/25 (0)	[112]
2004	EHH	CD and UC	Tissue	3/25 (12)	3/18 (17)	1/23 (4)	[113]
	H. pullorum			2/25 (8)	0/18 (0)	1/23 (4)	
	H. fennelliae			1/25 (4)	3/18 (17)	0/23 (0)	
	H. pylori			8/25 (32)	5/18 (28)	14/23 (61)	
2004	*Helicobacter*	CD, UC and IC	Tissue	0/11 (0)	1/20 (5)	0/37 (0)	[114]
2004	*E. coli*	CD and UC	Tissue	11/14 (79)	8/21 (38)	10/24 (42)	[157]
	AIEC			10/14 (71)	10/21 (48)	7/24 (29)	
2004	AIEC	CD	Tissue	7/63 (11.1)		1/16 (6.3)	[155]
2004	*E. coli*	CD	Tissue	12/15 (80)		1/10 (10)	[159]
2006	*E. coli*	CD and UC	Tissue	9/12 (75)	7/7 (100)	2/8 (25)	[160]
2007	AIEC	CD and UC	Tissue	8/13 (61.5)	11/19 (57.9)	4/15 (26.7)	[161]
2008	*Helicobacter*	CD	Faeces	17/29 (59)		1/11 (9)	[115]
	EHH			11/29 (38)		1/11 (9)	
	H. pylori			6/29 (21)		0/11 (0)	
	H. trogontum			4/29 (14)		1/11 (9)	
	H. canis			5/29 (17)		0/11 (0)	
	H. bilis			4/29 (14)		0/11 (0)	
	H. cinaedi			1/29 (3)		0/11 (0)	
2009	AIEC	CD	Tissue	14/27 (51.9)		4/24 (16.7)	[156]
2009	*Helicobacter*	CD	Tissue	32/73 (43.8)		43/92 (46.7)	[116]
	EHH			18/73 (24.7)		16/92 (17.4)	
	H. pylori			29/73 (39.7)		39/92 (42.4)	
	H. pullorum			8/73 (11)		6/92 (6.5)	
	H. canndensis			10/73 (13.7)		10/92 (10.9)	
2009	*Campylobacter*	CD	Tissue	27/33 (82)		12/52 (23)	[131]
	C. concisus			17/33 (51)		1/52 (2)	
	C. showae			3/33 (9)		0/52 (0)	
	C. hominis			2/33 (6)		2/52 (4)	
	C. gracilis			2/33 (6)		0/52 (0)	
	C. rectus			1/33 (3)		2/52 (4)	
	C. jejuni			1/33 (3)		3/52 (6)	
	C. ureolyticus			1/33 (3)		2/52 (4)	
2010	*Helicobacter*	CD	Tissue	32/77 (41.6)		23/102 (22.5)	[117]
	EHH			18/77 (23.4)		12/102 (11.8)	
	H. pylori			14/77 (18.2)		11/102 (10.8)	
	H. bilis			1/77 (1.3)		1/102 (1.0)	
	H. canis			2/77 (2.6)		0/102 (0.0)	
	H. hepaticus			2/77 (2.6)		2/102 (2.0)	
	H. trogontum			5/77 (6.5)		4/102 (3.9)	
2010	*Campylobacter*	CD	Faeces	39/54 (72)		10/33 (10)	[132]
	C. concisus			35/54 (65)		11/33 (33)	
2010	*C. concisus*	CD and UC	Saliva	13/13 (100)	5/5 (100)	57/59 (97)	[136]
2011	*Helicobacter*	UC	Tissue		32/77 (42)	11/59 (19)	[118]
	EHH				30/77 (39)	2/59 (3)	
	H. pylori				2/77 (3)	9/59 (15)	
2011	*C. concisus*	CD, UC and IC	Tissue	8/12 (66.7)	3/8 (37.5)	11/26 (42.3)	[133]
2011	*Campylobacter*	UC	Tissue		51/69 (73.9)	15/65 (23.1)	[135]
	C. concisus				23/69 (33.3)	7/65 (10.8)	
	C. ureolyticus				15/69 (21.7)	2/65 (10.8)	
	C. hominis				14/69 (20.3)	5/65 (7.7)	
	C. curvus				3/69 (4.3)	4/65 (6.2)	
	C. showae				4/69 (5.8)	0/65 (0)	
	C. jejuni				2/69 (2.9)	0/65 (0)	
	C. gracilis				1/69 (1.4)	0/65 (0)	

2011	*Campylobacter*	CD and UC	Tissue	12/15 (80)	11/13 (85)	18/33 (48)	[134]
	C. concisus			10/15 (67)	9/13 (69)	12/33 (36)	
	C. showae			1/15 (7)	2/13 (15)	2/33 (6)	
	C. hominis			1/15 (7)	1/13 (8)	3/33 (9)	
	C. ureolyticus			2/15 (13)	1/13 (8)	2/33 (6)	
	C. gracilis			1/15 (7)	1/13 (8)	0/33 (0)	
	C. rectus			0/15 (0)	1/13 (8)	0/33 (0)	
	C. jejuni			1/15 (7)	0/13 (0)	0/33 (0)	
2012	AIEC	CD and UC	Tissue	1/17 (5.9)	1/10 (10)	0/23 (0)	[167]
2013	*Helicobacter*	CD and UC	Tissue	4/29 (13.8)	1/13 (7.7)	5/42 (11.9)	[119]
	H. brantae			1/59 (3.4)	0/13 (0)	0/42 (0)	
	H. hepaticus			1/59 (3.4)	0/13 (0)	0/42 (0)	
2013	*Campylobacter*	CD and UC	Tissue	22/29 (75.9)	9/13 (69)	32/42 (76.2)	[119]
	C. concisus			13/29 (44.8)	4/13 (30.8)	16/42 (38.1)	
	C. curvus			2/29 (6.9)	0/13 (0)	3/42 (7.1)	
	C. gracilis			1/29 (3.4)	0/13 (0)	2/42 (4.8)	
	C. hominis			9/29 (31.0)	5/13 (38.5)	14/42 (33.3)	
	C. rectus			0/29 (0)	0/13 (0)	4/42 (9.5)	
	C. showae			9/29 (31.0)	5/13 (38.5)	9/42 (21.4)	
	C. ureolyticus			0/29 (0)	0/13 (0)	2/42 (4.8)	

CD: Crohn's disease; UC: Ulcerative colitis; IC: Indeterminate colitis; IBD: Inflammatory bowel disease; MAP: *Mycobacterium avium subspecies paratuberculosis*; EHH: *Enterohepatic Helicobacter*; *E. coli*: *Escherichia coli*; AIEC: Adherent-invasive *E. coli*.

juni) as the leading cause of gastroenteritis worldwide[129].

The role of *C. jejuni* in human disease has been long recognised and its prevalence in IBD investigated[130]. The main advance in the last decade has been the recognition of the importance of non-*jejuni Campylobacter* as human pathogens. Zhang *et al*[131] found a higher prevalence of *Campylobacter concisus* (*C. concisus*) DNA and IgG levels in newly diagnosed paediatric patients with Crohn's disease, even managing to culture *C. concisus* from a biopsy sample, indicating viability. Another study using faecal samples from newly diagnosed CD patients also found a significant association with *C. concisus*, 35 of 54 CD patients testing positive and only 11 of 33 healthy controls. This study also found that *C. hominis* was present in 13% of Crohn's samples, *Campylobacter ureolyticus* in 9%, *Campylobacter showae* (*C. showae*) in 4%, *Campylobacter gracilis* (*C. gracilis*) in 2% and *C. rectus* in 2%. Interestingly *C. gracilis, Campylobacter rectus* and *C. showae* were only detected in patient samples[132]. Similar results have been obtained in a number of studies in adult patients[119,133-136]. Mahendran *et al*[134] also showed an increased prevalence in UC, a finding supported by Mukhopadhya *et al*[135] who found *C. concisus* DNA in biopsy samples in 23/69 (33.3%) of UC patients compared to 7/65 (10.8%) of controls. This study also found *C. ureolyticus* to be in higher prevalence in UC patients. The most recent study found that although *Campylobacter* appear to be surprisingly common, with positive PCR in 33/44 IBD patients and 32/42 controls, there was no association with IBD[119]. A dominant serological antibody response to *C. concisus* has been documented in IBD patients indicating the prevalence of infection[137,138]. Specifically CD patients have been shown to recognise flagellin B, ATP synthase F α subunit and outer membrane protein 18 of *C. concisus*[137].

The origins of *Campylobacter* have led to a few researchers looking into the risks of developing IBD after acute gastroenteritis. A long term study published in 2009 documents the risk of developing IBD after acute infection with *Campylobacter* (*C. jejuni*) or *Salmonella*[139]. The findings indicated a significant increased risk in the exposed group for subsequently developing IBD, which has been supported by similar studies[140-142].

The pathogenesis of *C. jejuni* had been fairly well established prior to the last decade. *C. jejuni* has been used to induce colitis in rodent models and previous exposure correlated with disease severity[143]. The ability of *C. jejuni* to attach and invade the gut epithelium is well documented[144]. The newest discovery has been that *C. jejuni* can promote translocation of commensal luminal bacteria. This is a natural process thought to be essential for immunological tolerance and mucosal surveillance in the GI tract. Up regulation could affect the normal mucosal response to the intestinal microbiota leading to the chronic immune-mediated intestinal inflammation of IBD[145]

A number of studies have demonstrated the ability of *C. concisus* to colonise and adhere to intestinal epithelial cells, causing cell damage and microvillus degradation[146]. Man *et al*[146] comprehensively described the method of *C. concisus* attachment and invasion. They showed *C. concisus* to attach to the intracellular junction, disrupting barrier function - increasing permeability by causing a loss of tight junction proteins and decreasing transepithelial electrical resistance and to invade by a process mediated by polar flagellum[147]. Other non-*jejuni Campylobacters* have also been shown to be invasive and induce pro-inflammatory cytokines as well as producing a number of virulence factors such as haemolysins, cytolethal distending toxin and zonula occludens toxin[129,147-151]. These mechanisms could have an important bearing when one considers a causative role for this group of pathogens in IBD.

Adherent and invasive Escherichia coli

A specific pathogenetic group of *Escherichia coli* (*E. coli*), adherent-invasive *E. coli* (AIEC) have recently been extensively implicated in human IBD and are currently one of the most exciting players in the pathogen story. This

group are characterised by their ability to adhere and invade epithelial cells using actin microfilaments and microtubule recruitment. AIEC strains have been shown to be the cause of granulomatous colitis in boxer dogs and to induce granulomas, similar to early epithelioid granulomas, *in vitro*[152-154]. Similarly to the previously discussed bacteria, they have been documented to induce colitis in infected animals.

There is a growing body of evidence supporting the prevalence of AIEC in human disease. A number of studies initially showed a disproportionate increase in *Enterobacteria* as a whole[36,47]. When looking at AIEC organisms specifically, Darfeuille-Michaud *et al*[154] found them to be more prevalent in ileal Crohn's lesion tissue (36.4%) then controls (6.2%)[155]. This study also found that AIEC seemed to be rarely found in colonic tissue with 3.7% detected from Crohn's patients and 1.9% from controls, and none in UC specimens. This suggests a specific association of AIEC with ileal Crohn's. The findings of this initial study have been backed up by many researchers obtaining similar results[28,156-162]. Additionally, antibodies to the *E. coli* membrane protein C and the CD associated bacterial sequence I2 have been shown to not only be more prevalent in CD but also to be associated with more severe disease, with small bowel involvement, faster disease progression and increased need for surgical intervention[158,163].

The mechanism by which AIEC might induce colitis has been fairly well established towards the end of the decade. AIEC have type one pili and flagella that can bind to host adhesion receptor carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6)[164,165]. CEACAM6 has been shown to be more highly expressed in ileal CD tissue, to be increased after-γ or TNF-α stimulation and to be upregulated by AIEC itself[164,165]. AIEC have also been shown to possess long polar fimbriae and so can cross the mucosal barrier to access lymphoid cells[166]. They can then invade macrophages without inducing cell death, allowing them to replicate and continuously activate immune cells, triggering TNF-alpha release and granuloma formation, which are hallmarks of Crohn's disease. In fact the use of TNF-alpha antibodies has been shown to decrease the number of intramacrophagic bacteria, relating this to the success of anti-TNF therapy and further supporting the role of AIEC[167].

Other putative bacterial pathogens

In the last decade, there has been renewed focus in studying the role of various other bacterial strains in the aetiopathogenesis of IBD as well. The role of *Fusobacterium* was studied in mucosal biopsies of patients with IBD and was found to be significant compared to controls in a number of studies prior to this review period. More recently seropositivity to *Fusobacterium varium* (*F. varium*) infection was found to be higher in UC patients as opposed to controls with increased severity of disease in seropositive UC patients[168]. Further study has revealed the ability of *F. varium* to adhere to and invade colonic epithelial cells, increasing IL-8 and TNF-α secretion, providing a mechanism whereby *F. varium* infection may induce inflammation similar to that seen in IBD[169].

A similar association has also been found with *Klebsiella* infection, with Anti-*Klebsiella* antibodies found more commonly in IBD patients than in controls with the bacteria being implicated in disease relapses[170]. *Klebsiella pneumoniae* has recently been shown to increase the severity of colitis in mouse models, increasing COX-2, IL-1β, IL-6 and TNF-α expression and reducing tight junction associated proteins[171]. Colitis has been shown to be induced even in wild type mice highlighting the high pathogenic potential of this bacteria[172].

The role of *Salmonella* infection has been postulated from numerous studies that have documented the risk of developing IBD after acute *Salmonella* gastroenteritis[130,140-142]. When searching for a mechanism it has been found that a *Salmonella* virulence factor, the invasion-associated type Darfeuille-Michaud secretion system induces inflammation by activation of the NOD1/NOD2 signalling pathways[173]. This ties *Salmonella* nicely to the growing body of research into the genetics of IBD, supporting the role of the pathogen.

The potential role of *Yersinia* was proposed at the beginning of the last decade based upon the observed parallel increase in IBD and refrigeration, "the cold chain hypothesis"[174]. Similarly to *Salmonella* and *Campylobacter* there has been evidence that acute *Y. enterocolitica* infection increases the short and long term risk of developing IBD[175]. Despite some later successes in isolation *Yersinia* from IBD patients there has yet to be a compelling body of evidence coupling the prevalence of *Yersinia* with established IBD[176-178].

MODULATION OF THE GUT MICROBIOTA AS A TREATMENT OPTION IN IBD PATIENTS

The past decade has seen rapid and definitive strides in determining distinct changes in the gut microbiota in patients with IBD. The effects seem to be global, involving not only the physical composition of the principal components but also significantly altering their function. The role of individual pathogens in this complex milieu still needs to be elucidated. The proof of concept of "dysbiosis" as an important step towards developing IBD needs to be proven with therapeutic trials attempting to reverse the process.

Role of probiotics and prebiotics

Probiotics are beneficial microorganisms that, when ingested, may influence the gut microbiota composition, metabolic activity and immunomodulation to confer benefit to the host[179]. They can alter microbial diversity through competitive inhibition of other microbes, increase mucosal barrier function through the production of short chain fatty acids (SCFA) and interact with

intestinal DC to stimulate an anti-inflammatory response[180-183]. These probiotic strains must be of human origin, be non-pathogenic and have the intrinsic ability to survive the gastrointestinal transit in order to confer maximal benefit[184]. The most common probiotics used in the treatment of IBD have been *Lactobacillus* sp, *Bifidobacterium* sp, *Sacchromyces bouladrii*, *E. coli* Nissle 1917 and the probiotic combination VSL#3[185-192]. The strongest indication for the use of probiotics in IBD has been in the treatment of pouchitis in the post-operative setting in UC patients[193]. *E. coli* Nissle 1917 and VSL#3 have been found to be effective in preventing relapse and inducing remission in this setting[194-197]. The data is not very robust with respect to the role of probiotics maintaining remission in UC and a recent Cochrane Database System Review has not recommended its use[198]. The current body of evidence does not show any demonstrable benefit in patients with CD[199,200].

Prebiotics are non-digestible oligosaccharides that are selectively fermented in the colon into SCFAs and can alter microbial composition and activity and confer benefit to the host[201]. Examples include inulin, fructooligosaccharide (FOS), galactooligosaccharides and lactulose[202-207]. Prebiotics can selectively stimulate the growth of certain probiotics such as *Lactobacillus* and *Bifidobacterium*, decrease intraluminal pH and increase the production of SCFA, such as acetate and butyrate, which play an important role in epithelial and DC function[208,209]. SCFAs have also been found to have an anti-inflammatory effect[210]. In an open labelled trial FOSuse decreased the disease activity index in patients with active CD and resulted in increased faecal *Bifidobacterium*, but this benefit was not demonstrated in a subsequent randomized placebo controlled trial[206,207]. Another prebiotic inulin showed some promise in a randomized controlled trial in patients with UC and was also found to decrease inflammation in patients with pouchitis[203,204]. A couple of studies have also found a potential role of germinated barley foodstuff in maintaining remission in patients with active UC[211,212]. Lastly, some benefit was also accrued with the use of Ispaghula husk in patients with UC[213]. The important studies and their brief outcomes are summarized in Table 2.

Antibiotics

A couple of recent meta-analyses on antibiotics in IBD found that the use of antibiotics improved clinical outcomes of patients with IBD[214,215]. There is evidence that metronidazole and ciprofloxacin are useful in the treatment of CD and pouchitits[216,217]. Support for the use of antibiotics as the primary treatment in UC is less convincing, however, there are some studies which suggest that rifaximin and ciprofloxacin could be useful as an adjunctive treatment for UC[214]. The mechanisms through which antibiotics are thought to benefit patients with CD are through the inhibition of pathogenic bacteria or through reducing overall bacterial numbers. The main issues with antibiotic treatment include lack of understanding of which bacteria may be involved in the initiation of inflammation, lack of specificity and the potential for antibiotic resistance. There have been several trials in the past studying the specific role of anti-mycobacterials in the treatment of CD and this has been summarized in a European consensus document which has deemed the futility of such treatment[218].

Faecal transplantation

Faecal transplantation or faecal microbial therapy (FMT) as it is more commonly known is a technique in which stool is taken from a healthy surrogate and inserted into an unhealthy person, with curative intent[219]. The origins of faecal transplantation as a method of treating enteric pathology can be traced back for more than two millennia, when it was used as a traditional Chinese medicine to treat diarrhoea[220].

In recent times, FMT is perhaps best known for its potential role in treating *Clostridium difficile* (*C. difficile*) infectious diarrhoea[221]. After donor-faeces infusion in a group of patients infected with *C. difficile*, there was an alteration in the gut microflora with an increased faecal bacterial diversity, similar to that in healthy donors, with an increase in *Bacteroidetes* species and *Clostridium* clusters and a decrease in *Proteobacteria* species. This therapeutic benefit by FMT as documented in this trial, would theoretically have a beneficial effect in patients with IBD as well. The use of this form of intervention is still restricted to few exploratory trials. Donor faecal enemas were given to a group of ten children over five days with moderate to severe UC and resulted in 78% clinical response after a week and 67% with sustained response after a month in the nine children who could tolerate the treatment[222]. This was similarly documented in a subset of six adult UC patients who were treated over a period of 5 d. Complete reversal of symptoms was achieved in all patients by 4 mo, by which time all other UC medications had been ceased and at 1 to 13 years post FMT and without any UC medication, there was no clinical, colonoscopic, or histologic evidence of UC in any patient[223]. However, a single infusion in six adult patients with severe UC did not have a similar beneficial effect and the faecal microbiota changed to the donor phenotype in only 50% of those treated, suggesting that as opposed to treatment of *C. difficile* a prolonged treatment is indicated in IBD[224]. Although the data described above is certainly promising, there is clearly a need to move on from individual case reports and conduct more large scale randomised control trials before any benefit of FMT can be claimed with any certainty. Some concerns have also been raised regarding safety and side effects, with some IBD patients suffering mild side effects following FMT, and the obvious issues surrounding potential transmission of host infectious disease[224]. The efficacies of different administration techniques and dosing regimens for FMT also need to be refined and investigated. Literature to date describes a range of methods including colonoscopy, duodenal or gastric tubes and self-administered

Table 2 Probiotics and prebiotics in inflammatory bowel disease

Active component	Study	Design	*n*	Duration	Intervention	Result	Reference
Lactobacillus	CD remission	RCT	98	6 mo	*Lactobacillus johnsonii* LA1 4 × 10^9 cfu/d	No difference	[185]
	IBD		40	1 mo	*Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 supplemented yogurt	Anti-inflammatory effects	[186]
Bifidobacterium	Active UC	RCT	20	12 wk	*Bifido*-fermented milk [*B. breve*, *B. bifidum* and *acidophilus*] (1 × 10^{10}) or placebo	Decreased clinical activity ($P < 0.05$) decreased endoscopic/histological scores ($P < 0.01$)	[187]
	Active UC	Open label	12	4 wk	BGS 4.5 g/d	Decrease in clinical activity index ($P < 0.01$) and endoscopic scores ($P < 0.05$)	[188]
	C57BL/6 mice	Experimental	16	3 d	*B. bifidum* S17	Decrease in microscopic inflammation and reduction in inflammatory cytokines	[189]
E. coli Nissle 1917	UC remission		327	12 mo	200 mg *E. coli* Nissle 1917 or 1500 mg mesalazine/d	*E. coli* Nissle 1917 was equivalent to mesalazine in maintaining remission	[190]
VSL#3	UC remission	Open label	34	6 wk	VSL#3, 3.6 × 10^{12}, bacteria/d	ITT analysis demonstrated remission in 18/34 and response in 8/34	[191]
	Active UC	RCT	29	12 mo	VSL#3 450-1800 billion bacteria/d	Remission was achieved in 13/14 VSL#3 and 4/15 placebo ($P < 0.001$) Relapses within 1 yr of followup occurred in 3/14 VSL#3 and 11/15 placebo Endoscopic and histological score were significantly lower in VSL#3 *vs* placebo ($P < 0.05$)	[192]
Inulin	Active UC	RCT	19	2 wk	3 g/d mesalazine and either 12 g/d oligofructose-enriched inulin or placebo	Dyspeptic symptoms scale decreased significantly and an early reduction of calprotectin was observed in oligofructose-enriched inulin group	[203]
	Pouchitis	RCT	20	3 wk	24 g/d inulin or placebo	Reduction in inflammation, increase butyrate conc and decreased inflammation associated factors	[204]
Inulin and FOS	HLA-B27 rat model IBD			12 wk	8 g/kg body weight inulin or FOS	FOS increased *Bifidobacterium* spp. FOS and inulin reduced Clostridium cluster XI and *C. difficile* toxin gene expression correlating with a reduction of chronic intestinal inflammation	[205]
FOS	Active CD	RCT	103	4 wk	15 g/d FOS or placebo	No clinical benefit, despite impacting on DC function	[206]
	Active CD	Open label	10	3 wk	15 g/d	Significant reduction in Harvey Bradshaw index ($P < 0.01$) significant increase in faecal bifidobacteria conc. ($P < 0.001$) and modifies DC function	[207]

CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; RCT: Randomized control trial; FOS: Fructooligosaccharides; *E. coli*: *Escherichia coli*.

enema yet to date there is no clear evidence to support one method over any other. There is no doubt that manipulation of the gut microbiota could have enormous therapeutic potential and FMT will play an important role in its future.

CONCLUSION

The understanding of the aetiopathogenesis of IBD has undergone radical shifts in the past decade with the advent of modern molecular techniques that can characterize the gut microbiome more accurately and host genomic analysis that can explore the vast genetic universe of IBD. At the heart of the inflammatory process in IBD is "dysbiosis" of the gut microbiome, which may be driven by host genetics and environmental factors like diet. The next decade will help unravel the intricacies of the host immune defences that determine this intriguing host-microbiome ecology. The relationship of the genotype of the host and the extent to which it determines the composition of the microbiome needs to be elucidated. It will open the doors to more "personalized" therapeutic

interventions, which would encompass the host genotype and serotype, the disease phenotype, the gene expression profiles of the immune cells and the microbiome composition to decide the best strategy for treating patients with IBD. This will usher in a paradigm shift in patient management with a move away from standard generic therapy to a scientific, tailored approach based on the needs of individual patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Therapy with stem cells in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) affects a part of the young population and has a strong impact upon quality of life. The underlying etiology is not known, and the existing treatments are not curative. Furthermore, a significant percentage of patients are refractory to therapy. In recent years there have been great advances in our knowledge of stem cells and their therapeutic applications. In this context, autologous hematopoietic stem cell transplantation (HSCT) has been used in application to severe refractory Crohn's disease (CD), with encouraging results. Allogenic HSCT would correct the genetic defects of the immune system, but is currently not accepted for the treatment of IBD because of its considerable risks. Mesenchymal stem cells (MSCs) have immune regulatory and regenerative properties, and low immunogenicity (both autologous and allogenic MSCs). Based on these properties, MSCs have been used *via* the systemic route in IBD with promising results, though it is still too soon to draw firm conclusions. Their local administration in perianal CD is the field where most progress has been made in recent years, with encouraging results. The next few years will be decisive for defining the role of such therapy in the management of IBD.

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Key words: Mesenchymal stem cell; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Haematopoietic stem cell transplantation

Core tip: Treatments with mesenchymal and hematopoietic stem cells offer a potential that requires in-depth investigation. Existing studies are encouraging yet inconclusive. We are at a point of inflexion where these new therapies are seen to afford major curative potential. The coming years will be decisive. The information obtained from ongoing and future clinical trials may lead a revolution in inflammatory bowel disease management and its impact upon patients. Undoubtedly, as twenty-first century gastroenterologists, we must expand the scope of our specialty and seek multidisciplinary interaction for the benefit of our patients.

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INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are the two main conditions found in inflammatory bowel disease (IBD). The incidence and prevalence of IBD have gradually increased in recent years. It is estimated that in Europe 1.4 million and one million people may have UC and CD, respectively[1]. In the United States over 1.3 million people have IBD[1]. Furthermore, in the last few decades there has been an increase in the disease in low-incidence zones as South Korea, China, India, Iran

Lebanon, Thailand, the French West Indies, North Africa and Japan[2,3]. IBD poses an important health problem, since its worldwide incidence is increasing[1], the condition affects young people, and persists for life - exerting a strong impact upon quality of life, in the professional setting, and in patients' personal relations[4,5]. Furthermore, IBD is associated with considerable healthcare costs[6,7].

Although the etiology of IBD remains unclear, there have been significant improvements in our knowledge of its physiopathology, allowing advances in treatment and a change in the current therapeutic objectives. Before the introduction of anti-tumor necrosis factor-alpha (anti-TNFα) drugs, the management of IBD was centered on symptoms control. Drug substances (mesalazine, corticosteroids, thiopurines, methotrexate and cyclosporine) were used to induce and maintain disease remission. Although endoscopic disappearance of the lesions was known to occur in CD patients subjected to thiopurine therapy[8], the significance of mucosal healing was not known, and consequently did not constitute a therapeutic objective. The introduction in 1998 of anti-TNFα drugs for the treatment of CD improved the clinical outcomes of therapy, and mucosal healing was found to be associated with a good prognosis, fewer hospital admissions and surgical operations, and improved quality of life[9]. Later studies of both CD and UC showed mucosal healing to be a therapeutic objective[10-17]. In this context, current treatment focuses on achieving "deep remission" of IBD (symptoms control and endoscopic healing of the mucosal lesions)[14,15,18,19]. The purpose of treatment is to control the symptoms and the underlying intestinal inflammatory process (which is responsible for the progressive intestinal damage), and to restore normal intestinal function. Accordingly, in CD we aim to avoid structural intestinal damage, reduce disability over the long term, and improve patient quality of life. In the case of UC, we aim to reduce the percentage of colectomies and the incidence of colorectal cancer, and improve patient quality of life. All these strategies are aimed at changing the natural course of the disease.

However, despite the efforts being made to optimize use of the existing drugs, the current situation is far from ideal. Approximately one-third of all CD patients fail to respond to anti-TNFα therapy (primary non-responders)[9,11,20,21], and 10% of all CD patients do not tolerate or are primary non-responders to all the drugs used for treating the disease. Among the individuals who do respond to anti-TNFα treatment, one-third show transient loss of response and require either optimization or a switch to another biological agent (secondary non-responders)[22]. Furthermore, despite the new therapeutic strategies, the number of surgeries in CD remains stable[23]. Esophageal cancer (EC) is the second leading cause of intestinal transplantation in adults, as a consequence of the development of intestinal failure secondary to multiple surgical operations. In this context, disease recurrence in the graft is the norm[24,25]. Perianal disease can be a serious problem that proves difficult to control with the existing treatments, and refractory cases are subjected to aggressive surgery associated with a considerable psychological impact for the patient[26,27]. In addition to the above, it must be mentioned that IBD is becoming more common in the pediatric population, and patients now report to the clinic as adults with a history of multiple intestinal resections and immune modulating treatments with or without biological agents. In this population group, gastroenterologists predict that there may be a loss of response over the long term, and the future of these patients thus appears uncertain. Lastly, in some patients who do not respond to medical treatment, surgery is unable to solve the problem due to the location and extent of the lesions.

The situation is different in the case of UC, since in severe disease flare-ups and in patients resistant to medical treatment, costly surgery in the form of colectomy is a therapeutic option.

All this leads us to seek new treatment options. In recent years, efforts have increasingly focused on the study and use of cell therapies with T lymphocytes[28], tolerogenic dendritic cells[29] and both hematopoietic and mesenchymal stem cells (MSCs). The present review examines the role of stem cells in the control of IBD. An analysis is made of the physiopathological principles of stem cell therapy, the results of the studies carried out to date, and the future perspectives in this field.

PHYSIOPATHOLOGY OF IBD

Advances in our knowledge of the pathogenesis of IBD are the basis for the development of new treatments.

At present, IBD is regarded as the result of an abnormal host immune response to intraluminal antigens occurring in a genetically predisposed individual, with the production of chronic inflammation of the gastrointestinal tract, accompanied by tissue destruction. IBD is the consequence of complex interaction among genetic[30,31], environmental[1] and microbial factors[32], producing sustained inflammation at intestinal level, favored by alteration of the mucosal barrier and immune system defects[33].

Under normal conditions, the intestine presents minimal physiological inflammation, despite exposure to a large number of intraluminal microbial and food allergens, and the presence of a significant number of lymphoid cells (80% of all lymphocytes are located in the intestine)[33,34]. This situation is the result of complex mechanisms involving innate immunity[35-39], represented by the epithelial mucosal barrier, innate cell immunity (leukocytes, monocytes, macrophages and dendritic cells), and innate humoral immunity (*e.g.*, lysozyme, complement and interferons). While this innate response is not specific, it is able to differentiate so-called pathogen-associated molecular patterns, and to activate different intracellular mechanisms that condition or orientate the adaptive response. On the other hand, dendritic cells serve as the link between innate and adaptive immunity, presenting antigens to naïve Peyer's patch lymphocytes and inducing

their differentiation[40]. The Lymphocytes are the main effector cells of the adaptive immune response, and are activated at intestinal level to eliminate pathogenic antigens. Once the antigens have been eliminated, the lymphocytes are down-regulated, thereby maintaining intestinal homeostasis[38]. Lymphocytes are memory cells, and when they are exposed again to an already identified antigen, the immune response is both faster and more potent than at first exposure. The physiological mechanisms whereby the intestine differentiates pathogenic and non-pathogenic antigens, and the pathways involved in the correct maintenance of intestinal homeostasis, are not clear. We do know that a balance between regulatory T cells (Treg) and effector T cells (Th1, Th2 and Th 17) is essential for intestinal homeostasis[41,42].

It remains unclear whether the inflammatory process damages the mucosal barrier and thus allows penetration of the intraluminal antigens, thereby activating the inflammatory process, or whether mucosal barrier alteration is the primary event that in turn triggers the inflammatory response.

In recent years there have been major advances in the knowledge of the aspects that are believed to be involved in the development of IBD.

The genetic bases of IBD were recognized early in clinical practice in view of the increased incidence of the disease in homozygous twins, in first-degree relatives, and in certain ethnic groups[43-46]. At present, genome-wide association scan studies (GWAS) have identified over 100 loci related to the development of IBD[47]. A GWAS meta-analysis has revealed 77 genes associated to CD, the existence of shared genes between CD and UC[30], and 47 loci related to UC[31]. This suggests there are overlapping genes in both disease conditions which predispose to the development of IBD [genes with expression products that encode for the interleukin (IL)-23 pathway, transcription factors such as NK2, locus 3 (NKX2-3), SMAD3, STAT3, ZMIZ1 and c-REL], by encoding for proteins involved in the innate and adaptive immune responses[48-50]. Likewise, there are genes implicated in the development of CD such as NOD2, and genes that regulate autophagia[51,52], while other genes are specifically associated to UC, such as the genes located on chromosome 6p21 (related to the major histocompatibility complex), or to mucosal barrier integrity and defense[47]. On the other hand, genetic factors have been shown to be associated to the phenotype of the disease, its pattern, evolutive course and response to drug treatment[53-57]. Genetic disorders in IBD are multiple and complex, and unfortunately we are able to predict less than 25% of hereditary correlations in IBD[58]. The exception is represented by IL-10 receptor alteration, which is associated to severe CD, and seen in children refractory to treatment and with a good response to allogenic hematopoietic stem cell transplantation (HSCT)[59].

Epidemiological studies have shown that environmental factors are essential for the development of IBD, and could account for the increase in the presence of the disease in developing countries[1]. These environmental factors could exert a direct influence upon changes in the intestinal microbiota, or on the appearance of new intraluminal antigens from foods or environmental toxic agents. Diet and antibiotics are the most important determining factors of bacterial diversity in the gastrointestinal tract, and their modification runs parallel to the socioeconomic development of countries[60]. The microbiota plays a key role in the development of IBD among genetically susceptible hosts[61]. In this regard, a recent publication addresses the possible infectious origin (produced by an unknown bacterium) of a case of colitis with histological data indicating chronicity and the presence of granulomas. The condition is considered idiopathic, and occurs in patients subjected to umbilical cord allogenic hematopoietic cell transplantation[62]. This opens a door to the future search for microbial agents in similar disease conditions characterized by an unknown etiology.

Lastly, intestinal homeostasis is lost in IBD as a result of defects in the intestinal epithelial barrier and/or imbalances between Treg and effector T cells (Th1, Th2, Th17) - giving rise to an inappropriate immune response to harmless intraluminal antigens (components of the diet) or intraluminal bacteria[42,63].

It is currently accepted that the predominant inflammatory profile in CD involves a type Th1 and Th17 activated $CD4^+$ lymphocyte response, with an increase in interferon-γ (IFN-γ), TNFα, IL-17 and IL-22[64]. In UC, the cytokine profile is similar to that of the natural killer (NK) cell mediated Th2 response, producing IL-5 and IL-13. This latter interleukin is crucial for the development of UC, exerting cytotoxic action against the epithelial cells and positive feedback upon the NK cells, resulting in tissue damage[64,65].

Improved knowledge of the pathways leading to inflammation, and of the implicated cytokines, cells and adhesion molecules, have allowed the identification of many therapeutic targets upon which to act. Until the causal agent can be identified, these are the advances which can help us in the search for new drug substances.

Stem cell therapy aims to modify the immune response of patients with IBD and repair the tissue damage caused.

STEM CELL THERAPY

Stem cells are characterized by asymmetrical division, giving rise to a cell with the same properties as the original cell (self-renovation), and another cell of multilineage potency that can differentiate in one way or another depending on environmental conditions.

There are different types of stem cells, depending on their origin and functions[66].

Embryonic stem cells

These are pluripotent cells obtained from embryos, and which can produce all the tissues derived from the three embryonic layers both *in vitro* and *in vivo*.

Table 1 Two types of stem cell therapy are currently used for the treatment of inflammatory bowel disease

Type of therapy	Mechanisms of action
Hematopoietic stem cells	Autologous transplant: Elimination of reactive T lymphocytes (lymphoablation, new reconstitution of the immune system of the patient with more tolerogenic naïve lymphocytes). The genetic predisposition of the patient is not modified Allogenic transplant: Replacement of the immune system with the donor immune system, correcting patient genetic predisposition. Not accepted due to high morbidity-mortality
Mesenchymal stem cells (autologous or allogenic)	Systemic and local administration: Immune-modulating and trophic action

Multipotent adult stem cells

These cells are found in all body tissues, where they may have reparatory functions. Hematopoietic stems cells (HSCs) and MSCs belong to this category. Both types are found in bone marrow, though MSCs can be obtained from different tissues (bone marrow, adipose tissue, umbilical cord, placenta, *etc*.), do not produce hematopoietic cells, and can differentiate towards cells belonging to the mesenchymal lineage.

Induced pluripotent stem cells

These are defined as artificial pluripotent stem cells[67] that can be generated from somatic cells following the introduction of reprogramming factors (OCT314, SOX2, KLF4, c-MYC, NANOG and LIN28). These cells acquire the same properties as pluripotent stem cells, and their phenotypic differentiation can be redirected according to the culture media used. These cells pose no ethical problems, since they are not of embryonic origin. As a result, they are currently under study for the development of organs and the repair of tissues carrying the genome of the sick patient[68].

The use of embryonic stem cells and induced pluripotent stem cells has been associated to the development of teratomas following transplantation. This fact, and the present impossibility of eliminating undifferentiated cells produced as a result of division, poses a serious problem for clinical use[69].

Two types of stem cell therapy are currently used for the treatment of IBD: hematopoietic stem cell therapy and MSCs therapy (Table 1). Most treatment evaluations are made in CD patients, since in UC patients surgery is an option in the case of resistance to therapy.

HEMATOPOIETIC STEM CELL THERAPY IN IBD

Hematopoietic stem cells are immature cells found in bone marrow, the bloodstream and the umbilical cord. They present glycoproteins as $CD34^+$, $CD\ 90^+$, $CD38^-$, CD133 at surface level and can differentiate towards cells pertaining to hematological cell lines[70]. In clinical practice, HSCT was initially used to treat hematological malignancies. Subsequently, as a result of experimental studies in animals[71,72] and the clinical improvement experienced by patients with immune-mediated diseases (IMDs) subjected to HSCT due to malignant conditions[73,74], HSCT has become increasingly used in selected patients with IMDs refractory to conventional therapy[75,76]. The treatments used at present in application to IBD aim to control the inflammatory process but do not act upon the origin of the disease. The therapeutic objective of autologous HSCT would be to restore the primary immune system of the patient (resetting of the immune system), after using chemotherapy to eliminate self-reactive T lymphocytes (lymphoablation) and memory cells, which would constitute the effectors of the immune dysregulation observed in CD[77], thereby inducing antigen tolerance over the long term. Based on studies carried out in other IMDs[78,79], the changes of the new immune system could be the result of reactivation of thymus gland activity (with the restoration of new polyclonal T cells) and the *de novo* induction of Treg derived from the thymus, which would be essential for restituting tolerance of harmless antigens[80-82]. Genetic factors are crucial in the development of IBD, and are not modified by autologous HSCT. IBD might reappear after exposure to triggering antigens, and the body would probably respond in the same way. However, and in the worst of cases, the available therapies may be prescribed earlier in an attempt to change the natural course of the disease, or while waiting for the development of new treatments.

In any case, allogenic HSCT can correct the genetic defects of the patient by conforming a new immune system (that of the donor), associated to ablation of the immune cells of the recipient[77]. This transplant strategy is currently not accepted as primary treatment for IMDs, due to its high associated mortality and complications rate.

In 1995, an international committee produced guidance with the criteria and protocols for performing HSCT in patients with severe IMDs, including IBD[83]. The committee recommended autologous HSCT instead of allogenic transplantation, due to its lower risk and toxicity.

HSCT in IMDs is carried out following the protocol used in the treatment of hematological malignancies. In autologous HSCT, the hematopoietic stem cells are obtained from peripheral blood in 95% of all cases. To this end, a first mobilization step is carried out, stimulating the production and release of stem cells from the BM towards the peripheral blood compartment. The treatment scheme most widely used in CD is cyclophosphamide 1.5-2 g/m^2 and granulocyte colony stimulating factor (G-CSF) 10 μg/kg per day[84-86]. Peripheral blood stem cells are then collected through apheresis followed by cryopreservation until HSCT is performed. Before reinfusing the hematopoietic stem cells, some work groups use local protocols to purify cell culture, selecting $CD34^+$ cells or selectively eliminating lymphocytes by means

of monoclonal antibodies (anti-CD52, anti-CD3, anti-CD19 or anti-CD20)[84]. Other groups do not eliminate lymphocytes[85]. Finally, the conditioning stage (elimination of self-reactive T cells) is carried out, using cyclophosphamide and anti-thymocyte immunoglobulin[86], and hematopoietic cell infusion is carried out at the end of this stage.

The conditioning regimens used vary according to the autoimmune disease and the treating center[87]. Non-myeloablative conditioning regimens have been specially designed for IMDs. In the case of IBD, lymphoablation is performed without irreversible destruction of the hematopoietic cells of the BM, and marrow function could be recovered without the infusion of hematopoietic stem cells. Reinfusion is performed to shorten the duration of BM aplasia. The reasons for not using myeloablative regimens would be safety in first place, since lymphoablative protocols present fewer complications and a lower mortality rate. On the other hand, IBD recurrence after autologous HSCT is highly probable, since the immune system of the patient is regenerated. In turn, following a very aggressive initial phase, some IMDs undergo spontaneous remission for reasons that are unknown, and in these patients such aggressive therapy would be unnecessary[81].

Autologous HSCT in EC

The first case reporting the efficacy of autologous HSCT in the control of CD was published in 1993, and corresponded to a patient with non-Hodgkin lymphoma[88]. Subsequently, isolated cases of IBD improvement in patients receiving autologous HSCT for malignancies were published[89-92]. In 2003, the Chicago group published the first series of CD patients subjected autologous HSCT as primary treatment for IBD[93]. A later publication by this same group[84] reported the results of autologous HSCT (phase I trial) in 12 patients with CD and EC activity index (CDAI) scores of 250-450, refractory to conventional treatment, including infliximab. Eleven patients presented disease remission, defined by CDAI < 150, with a mean follow-up of 18.5 mo (range 7-37 mo). In 2008, Cassinotti *et al*[85] published the clinical results of autologous HSCT in four CD patients. Clinical and endoscopic remission was observed in three subjects after 16.5 mo of follow-up (phase I - II trial). Unlike the Chicago group, these authors do not perform $CD34^+$ cell selection before hematopoietic stem cell infusion. Since then, some isolated cases as well as larger patient series have been published in which the primary objective of HSCT has been the control of refractory CD[82,94-96]. In the series published by Hasselblatt *et al*[96], 56% of patients achieved endoscopic healing of mucosal lesions after cell transplantation - these data being similar to those obtained in the phase III trials with anti-TNFα[9-11]. The study carried out by Oyama *et al*[84] reported improvement of CD following the cell mobilization phase with low dose cyclophosphamide and G-CSF, and suggested that the improvement may have been due to the action of chemotherapy rather HSCT. However, Cassinotti reported worsening of CD in three of the four patients in the period between stem cell harvesting and transplantation. The phase III Autologous Stem Cell Transplantation International Crohn's Disease (ASTIC) study, sponsored by the European Crohn's and Colitis Organisation (ECCO) and the European Group for Blood and Marrow Transplantation (EMBT), was designed to clarify this issue. The patients included in this study were randomized to two treatment arms: mobilization chemotherapy with G-CSF and autologous HSCT in 30 d *vs* mobilization chemotherapy with G-CSF and conditioning with autologous HSCT after 13 mo. The preliminary results have been presented in the ECCO of 2013, concluding that HSCT appears to be effective in CD patients, affording endoscopic improvement of the lesions. However, it involves a risk of adverse effects, and the end results referred to the trial objective are still awaiting analysis[97].

It is difficult to know the precise results of autologous HSCT in CD, due to the few patients treated to date. In the series published by Burt *et al*[94], which is the most numerous to date, all patients (n = 24) entered remission (CDAI < 150) after transplantation. Over subsequent follow-up, the percentage of patients in remission without the need for CD treatment was 91%, 63%, 57%, 39% and 19% after one, two, three, four and five years, respectively. Regardless of the medication taken, the percentage of patients annually in remission in the 5 years after transplantation was 70%-80%. Eighty percent of patients were in corticosteroid-free remission each year, in the 5 years after transplantation. Regarding the safety of the procedure, infectious complications during the first year were the most significant problem, though mortality was zero (one patient died in an accident).

The overall safety of HSCT in the treatment of autoimmune diseases can be assessed from the two large recently published registries: that of the EMBT, and the registry of the British Society of Blood and Marrow Transplantation (BSBMT). The results of the observational study of the EMBT, documenting the course of 900 patients subjected to autologous HSCT between 1996-2007, showed the 5-year survival rate to be 85%, with a patient disease-free rate of 43% - though there were important variations depending on disease type. In the multivariate analysis, mortality was found to be related to the experience of the center ($P < 0.003$) and type of autoimmune disease ($P < 0.03$). An age of less than 35 years ($P < 0.004$), transplantation after the year 2000 ($P < 0.0015$), and type of autoimmune disease ($P < 0.0007$) were associated to a disease-free patient course[87].

In 2012, Snowden *et al*[86] published the results on HSCT in patients with IMDs documented in the BSBMT registry between 1997-2009 (70 transplants in 69 patients: 55 autologous cell transplants and 15 allogenic transplants). The survival rate in the case of autologous transplants was 85% in the first year and 78% after 5 years. The corresponding figures for allogenic transplantation were 87% and 65%, respectively. The disease-free rate for autologous transplants was 51% in the first year and

33% after 5 years. The corresponding percentages for allogenic transplantation were 80% and 65%, respectively. There were no differences in mortality in the two groups. The leading cause of death was infection, while age was strongly correlated to survival (95% survival after 5 years among patients between 18-39 years of age).

Autologous HSCT may be a valid option in patients with treatment-refractory CD. This therapy must be provided at specialized centers where correct patient screening is performed, and in which complications referred to both transplantation and autoimmune disease are known and can be treated. Further studies are needed to determine the precise role of this procedure in CD treatment.

Allogenic HSCT in IBD

No descriptions were found in the literature of allogenic HSCT as primary treatment for CD until 2009, when this technique was first used to treat a patient with mutation of the *IL10RA* and *IL10B* genes (encoding for proteins ILR10R1 and ILR102, which form part of the IL-10 receptor). This mutation was identified in homozygosis in three children from the same family that developed proctitis early (at 3 mo of age), with severe perianal disease refractory to treatment. Following allogenic HSCT, sustained CD remission was observed[59].

This treatment is not currently accepted as primary therapy for IBD and, with the exception of the aforementioned case, the results found in the literature correspond to patients with neoplastic disease associated to IBD, in which the course of the latter changed after HSCT. The most numerous allogenic HSCT series to date is that published by Ditschkowski *et al*[98]. In this series, 11 patients (7 with CD and 4 with UC) underwent allogenic HSCT due to hematological malignancies. All patients were subjected to myeloablative treatment and total body irradiation (except in two cases). One patient died of sepsis, while the rest were found to be in complete neoplastic disease remission after 34 mo of follow-up, with no clinical relapse of IBD.

Lopez-Cubero *et al*[99] previously described 6 CD patients diagnosed with leukemia and subjected to allogenic HSCT. Five of them had active CD before transplantation, and two had sclerosing cholangitis. All of them received cyclophosphamide in the total body irradiation scheme. One patient died of sepsis three months after transplantation. Four of the remaining 5 patients remained symptom-free during a period of 54-183 mo after transplantation. The patients with a clinical diagnosis of primary sclerosing cholangitis showed improvement in alkaline phosphatase levels.

Similar findings have been described in a patient with CD and acute myeloid leukemia subjected to allogenic HSCT[100].

This procedure is not currently recommended in IBD, except in very specific cases such as mutation of the *IL-10* gene commented above. Its efficacy and risks have been described in the above section.

MSC THERAPY IN IBD

MSCs, also known as stromal cells, are adult pluripotent cells initially described by Friedenstein *et al*[101], who isolated them from bone marrow. They are able to adhere to plastic surfaces, and can differentiate *in vitro* into chondrocytes, osteoblasts and adipocytes. The International Society for Cellular Therapy established the minimum criteria that must be met in order to classify a stem cell as an MSC[102]: (1) adherent to plastic under standard culture conditions; (2) express CD105, CD73 and CD90; (3) lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen (HLA)-DR; and (4) differentiate to osteoblasts, adipocytes and chondroblasts *in vitro*.

These cells have been identified in bone marrow, adipose tissue, connective tissue, umbilical cord and placenta, and it is known that they are found in "niches" in different tissues - including the intestine[103,104]. In most clinical studies and in human therapy, MSCs are obtained from bone marrow, adipose tissue and umbilical cord. Adult bone marrow has been widely used to obtain MSCs for clinical purposes, though their presence in adult marrow is low (0.001%-0.01%), extraction is difficult, and the procedure also poses risks for the donor. Furthermore, the differentiation capacity of these cells decreases with donor age, and expansion must be performed in culture media prior to administration of the cells. As a result, alternative MSC sources have been explored[105]. A larger number of MSCs can be obtained through liposuction; *in vivo* expansion is not required; cells can be administered directly; and their properties are similar to those of MSCs obtained from bone marrow[106-108]. A recent study published by Melief *et al*[109] shows that both types of cell have similar immune modulating functions, though MSCs of adipose tissue origin present a different cytokine secretion profile, and their immune modulating effects are more potent that those of MSCs obtained from bone marrow.

The use of MSCs is based on their potential capacity to repair damaged tissues and inhibit inflammation and fibrosis[110,111]. In addition, the administration of MSCs of both autologous and allogenic origin requires no conditioning phase, since these cells are not immunogenic: they express low levels of class I HLA antigens at surface level and do not express type Ⅱ HLA antigens or T cell coactivators[112]. However, in immunocompetent mice it has been seen that the half-life of these cells is notoriously shorter following second exposure. This suggests they do not evade the immune system entirely, and can be rejected[113-115]. These properties have defined MSCs as potentially useful for the treatment of autoimmune diseases and in those processes in which tissue repair is needed[112].

The action mechanism is based on cell contact and paracrine action involving the release of soluble factors (Figure 1).

As regards the immune regulating properties of the

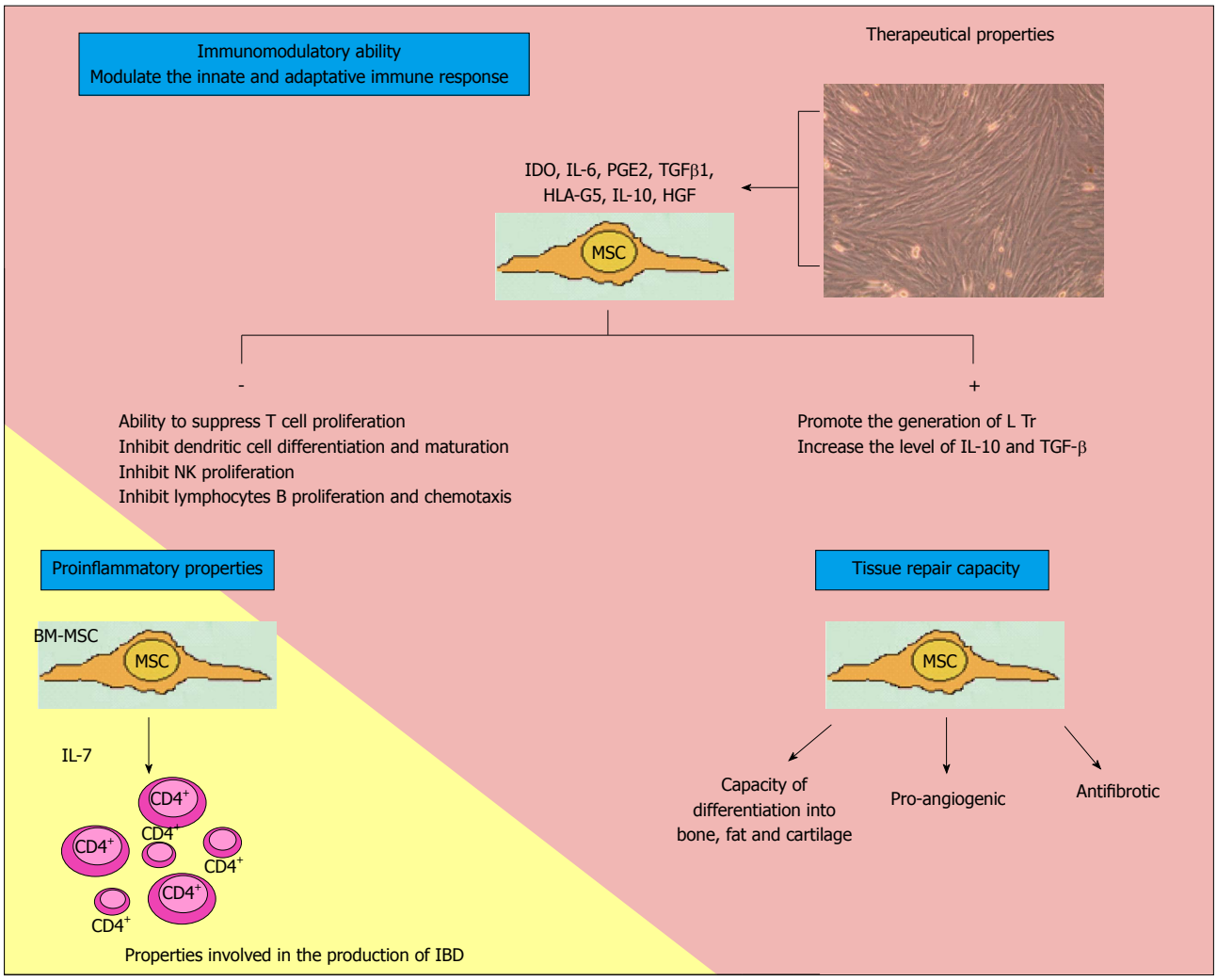

Figure 1 Characteristics of multipotent mesenchymal cells. MSCs: Multipotent mesenchymal cells; IDO: Indoleamine 2,3 dioxygenase; IL: Interleukin; PGE2: Prostaglandin E2; TGFβ1: Transforming growth factor beta-1; HLA-G5: Human Leucocyte Antigen G5; HGF: Human growth factor; NK: Natural killer; Tregs: Regulatory T cells; BM-MSC: Bone-marrow-mesenchymal stem cell; $CD4^+$: Activated lymphocytes CD4; IBD: Inflammatory bowel disease.

cells, they are capable of regulating both innate and adaptive immunity[116]. Cell culture studies have shown that MSCs are able to suppress T cell proliferation[113]. However, MSCs do not possess intrinsic immunosuppressive capacity; instead, such capacity is acquired when cells are stimulated by proinflammatory cytokines such as INFγ, TNF-α and interleukin-1β[117]. Duijvestein *et al*[118] have shown MSC activation by INFγ to increase the immunosuppressive capacity of cells and their *in vivo* therapeutic efficacy in mice with colitis induced by trinitrobenzene sulfonate.

MSCs produce a range of factors that have been implicated in immune modulating effects, such as indoleamine 2,3-dioxygenase, IL-6, IL-10, prostaglandin E_2, transforming growth factor-β1, nitric oxide, heme oxygenase-1 and HLA-G5[109]. Through the production of PGE, MSCs inhibit IL-2 production and T cell proliferation, with stimulation of the production of T helper lymphocytes[119]. IL-10 and IL-6 act upon macrophages and monocytes, blocking their differentiation towards dendritic cells[120,121]. They also suppress NK cells, induce polymorphonuclear cell and cytotoxic T cell apoptosis, and promote the generation of regulatory T cells[122]. These interleukins also inhibit B lymphocyte activation[123]. Upon coming into contact with activated T cells, MSCs induce the production of IL-10 and HLA-G5[124]. In short, they inhibit innate immunity by blocking the differentiation and maturation of monocytes towards dendritic cells, inhibiting NK cells, and inducing neutrophil apoptosis. In induced colitis in mice, MSCs block T cells and increase regulatory T lymphocyte population, thereby modulating acquired immunity.

However, MSCs can acquire proinflammatory properties, depending on biological environment[125]. In this context, Nemoto *et al*[126], in a murine model, have shown that bone marrow MSCs constitute the main source of IL-7 production, and may play an etiopathogenic role in IBD by forming a niche for colitogenic $CD4^+$ memory T cells in bone marrow. If these findings are confirmed in humans, anti-IL-7 treatment might become a therapeutic target.

MSCs are able to repair tissues, stimulate angiogenesis

and prevent fibrosis. Healing of the intestinal lesions in IBD is the result of control of the underlying inflammatory process, and of repair mechanisms that restore the integrity of the epithelial barrier and repair intestinal damage. The initial applications of MSCs in regenerative medicine were focused on their multilineage differentiation capacity. However, recent reports have demonstrated that most of the biological effects of MSCs are mediated by paracrine mechanisms involving the secretion of cytokines, chemokines and growth factors[127-129]. Indeed, there are several observations of positive MSC biological effects in numerous disease models despite a lack of MSC differentiation and long-term engraftment into damaged or diseased tissue. These effects include a reduction of inflammation, apoptosis and fibrosis, improved wound healing and regeneration[130-133]. In addition, MSC biological effects also may be exerted by the induction and stimulation of endogenous host progenitor cells to improve the regenerative process[134-136].

When administered *via* the systemic route (intraarterial or intravenous) or locally, the objective is to ensure that MSCs reach damaged tissue. However, the correct dose and administration route remain to be defined, and may even differ depending on the type of lesion involved[69]. Forty-eight hours after systemic administration in animals, the cells are found to be located in the lung and liver[137], while after 9-21 mo they are detected in the lungs, liver, pancreas, spleen, kidneys and skin[69,138].

The engraftment rate in these tissues is 0.1%-2.7%, and is similar for both autologous and allogenic MSC transplantation[138].

Another aspect to be taken into consideration is that the drugs used to treat IBD do not appear to alter MSC function[139].

MSCs were first used in clinical practice to treat graft-*vs*-host disease (GVHD) in a patient with acute lymphoid leukemia refractory to therapy. Improvement was notable, and the patient remained free of gastrointestinal lesions after one year[140].

The company Osiris has created an MSC product based on bone marrow from healthy donors (Prochymal), and has designed a phase Ⅱ open-label study on the treatment of acute GVHD. Two treatment arms were defined: one group received high-dose MSCs (8 million MSCs/kg), while the other group received low-dose MSCs (2 million MSCs/kg). Two infusions were administered in both groups, spaced three days apart. The following results were obtained: 24 out of 31 patients showed complete clinical response (absence of GVHD symptoms in skin, liver and gastrointestinal tract), while 5 showed partial response[141]. However, a phase Ⅲ study involving a similar group of patients failed to obtain significant results in relation to the primary endpoint[142].

At present, MSCs are being used for repair and immune modulating purposes in clinical trials on the treatment of cardiovascular and neurological disease, in the treatment of autoimmune disorders, and in the management of post-radiotherapy tissue damage[128,130,132,135,136,143]. Laboratory-based studies reporting antineoplastic activity have recently been published[144].

Two types of treatment with MSCs have been developed in IBD: systemic administration of MSCs (intraarterial or intravenous) for the control of intestinal inflammatory disease, and the local administration of MSCs in perianal fistulizing CD.

Systemic MSCs therapy in IBD

The use of MSCs for IBD treatment is based on their reparatory and immune modulating properties.

Several phase Ⅰ-Ⅱ studies involving autologous and allogenic MSCs have been published in recent years on the treatment of IBD.

In 2006, Onken *et al*[145] published an abstract on MSCs used to treat 10 patients with active CD (CDAI > 220, C-reactive protein ⩾ 5 mg/L) refractory to treatment with corticosteroids, immune modulators and infliximab. Use was made of MSCs obtained by bone marrow puncture from healthy donors (Prochymal, Osiris). The patients were randomized to two groups, both of which received two intravenous doses of MSCs, spaced one week apart. One group received high-dose MSCs (8 million MSCs/kg), while the other group received low-dose MSCs (2 million MSCs/kg). The primary endpoint was percentage clinical response, defined as a reduction in the CDAI score of ⩾ 100 points. Four weeks after the end of treatment, and as a secondary endpoint, improvement was observed in the inflammatory bowel disease questionnaire (IBDQ) scores during the same period. Nine patients completed the study. All subjects presented a mean reduction in CDAI score of 105 points on day 28. The CDAI and IBDQ scores decreased to a greater extent in the high-dose group, though statistical significance was not reached[145].

A phase Ⅲ, randomized and placebo-controlled trial was started by Osiris in 2007 (protocol 603, NCT00482092 clinicaltrials.gov)[146]. The aim was to include a large number of patients with active CD (CDAI score 250-450; PCR ⩾ 5) and a history of intolerance or resistance to corticosteroids, immune modulators and biological drugs. The patients were randomized to four intravenous MSC infusions in two weeks. One group of patients received a total of 600 million cells (low dose), while the rest received 1200 million cells (high dose) or placebo. The primary endpoint was remission on day 28, while the secondary endpoints were clinical response, improvement in quality of life, and reduction of the number of draining fistulas. In March 2009, with 207 patients enrolled, the trial was suspended because of a high placebo response. Subsequently, the United States Food and Drug Administration authorized the reopening of the study, though no results are currently available[147].

More recently, phase I studies have been published that allow us to know the safety and possibilities of this type of therapy. In 2010, Duijvestein *et al*[148] published the results of a phase I trial with intravenous MSCs administered to 10 patients with CD refractory to cortico-

steroids, immune modulators and anti-TNFα drugs. The cells were obtained by bone marrow puncture of CD patients, and 9 subjects received two doses of 1-2 million cells/kg body weight, spaced one week apart. This study showed the autologous MSCs of CD patients to be functionally analogous to those of healthy donors, maintaining the same immune modulating properties, and without experiencing alterations due to the drugs administered to the patient. In relation to CD, the benefits obtained were few: only three patients showed improvement, with a reduction of CDAI ⩾ 70. The procedure proved safe, with no significant side effects.

In 2012, Liang *et al*[149] reported the results obtained in 7 patients with IBD (4 with CD and 3 with UC). In three cases MSCs were obtained from the bone marrow of healthy donors, and in four cases from umbilical cord. The dose administered consisted of one million cells/kg *via* the intravenous route. All the patients maintained their medication (corticosteroids and immune modulators) after the infusion of MSCs. Five subjects showed remission, and the latter was maintained for over 24 mo in two of them. Two CD patients and one UC patient showed improved endoscopic indices, and in all three of these subjects biopsies revealed a decrease in the extent of IBD and in intensity of lymphoid infiltrate. Side effects were mild: one patient experienced facial flushing for 6 h after the infusion, while another experienced insomnia during the first night following infusion. One patient developed febricula and worsening of diarrhea. These symptoms disappeared without medication of any kind, and no other side effects were observed.

Regarding the safety of MSC treatment, clinical trials conducted in human subjects have found the therapy to be safe, with no toxic effects or generation of ectopic tissue. The most commonly reported side effect was transient fever[140,145,146,148-150]. MSCs may be infected with viruses (*e.g.*, cytomegalovirus, herpes virus), and a case of infection with Epstein-Barr virus has been reported in a patient with a lymphoproliferative process previously subjected to bone marrow conditioning treatment, followed by the administration of MSCs due to an episode of GVHD[151,152]. As mentioned, a possible case of bacterial infection transmitted by umbilical cord hematopoietic stem cells has recently been reported[62].

The above data are promising, yet the number of treated patients is small, the source of MSCs diverse, and the regimens and doses different. It is therefore not possible to draw firm conclusions at this time. Strictly protocolized trials need to be conducted to draw valid conclusions.

Local MSCs therapy in fistulizing EC

MSCs treatment for perianal CD has been carried out using MSCs obtained from bone marrow or adipose tissue. We do not know exactly the mechanism of how MSCs work in perianal fistulas. As we explained before, MSCs and expanded allogeneic adipose-derived stem cells (ASCs) have the potential to control systemic and local inflammatory pathways by suppressing the proliferation of activated lymphocytes. By controlling the local inflammatory process they could induce cicatrization of the fistula due to reparative properties.

Regarding the first cell source, the experience gained corresponds only to an Italian study published in 2008[153]. The authors treated 9 CD patients - 8 with complex perianal fistulas and one with multiple enterocutaneous fistulas - using MSCs from bone marrow, serially injected into the fistular canal every four weeks, with an average of four doses in total. Remission was achieved in 7 of the 10 patients, while improvement was noted in the remaining three, with marked and significant reductions in the perianal disease activity index and CDAI scores. The findings were confirmed by endoscopy and magnetic resonance imaging (MRI), and evident improvement of the rectal inflammation was also evidenced. The authors postulated that part of the effect may have resulted from stem cells reaching the lymph nodes, influencing T lymphocyte differentiation and[154] apoptosis.

Regarding ASCs, a large number of studies have been published over the last decade. The first such study dates back to 2003[155]. Based on the previously reported myogenic, adipogenic or chondrogenic differentiation potential of these cells[156], ASC injection into a recto-vaginal fistula of a CD patient resulted in sealing of the fistula. From this point onwards, a series of studies were conducted to explore the usefulness of these cells in this field. The first publication was a phase I study[157] carried outperformed in 2005 to evaluate the safety and viability of the treatment. ASCs were inoculated in 8 complex fistulas in 5 CD patients, with evaluation of the response after 8 wk. The results showed re-epithelization of the external fistular orifice, with complete sealing of 6 fistulas (75%) and a partial decrease in suppuration in the remaining two (25%). No serious adverse effects were reported. Biopsies obtained from the zone in two patients after 7 and 12 mo showed no evidence of dysplastic transformation.

Following the results obtained, a new phase Ⅱ, randomized, open-label, controlled multicenter study was published in 2009[158]. This study included 35 patients with complex cryptogenic fistulas, including 14 associated to CD, randomized to either fibrin glue or fibrin glue plus 20 million ASCs. Evaluations were made after 8 wk and one year, with the possibility of administering a second dose of 40 million ASCs in cases showing failure in week 8. Sealing of the fistula, defined as closure of the external orifice and the absence of suppuration in response to digital pressure, was recorded in 11 patients (46%) in the ASC treatment arm, *vs* in two patients (8%) in the non-cell therapy arm, after 8 wk. In turn, following both doses, sealing was recorded in 17 patients (71%) in the ASC treatment arm and in four patients (16%) in the fibrin glue treatment arm (RR = 4.43, 95%CI: 1.74-11.27, $P < 0.001$). Effectiveness was found to be similar on considering a cryptoglandular origin of the fistulas *vs* fistulas associated to CD, though statistical significance was not reached in this latter subgroup due to the small number

of patients involved. No adverse effects related to ASC use were reported.

The results of the FATTI phase Ⅲ, randomized, single-blind, multicenter clinical trial were published in 2012[159]. In this study IBD constituted an exclusion criterion, and a total of 200 subjects diagnosed with simple cryptoglandular fistulas (confirmed by MRI) were included. The patients were randomized to three groups: 20 million ASCs (group A), 20 million ASCs with fibrin glue (group B), and fibrin glue with placebo (group C). The possibility existed of a second dose of 40 million ASCs in week 12. The primary endpoint was fistular sealing (re-epithelization of the external orifice, with absence of drainage and no collections as evidenced by MRI) in week 12 and in weeks 24-26. Of the 200 randomized individuals, 183 received treatment, and 165 completed the study [per protocol (PP) analysis]. After 12 wk fistular sealing was observed in 26.5%, 38.33% and 15.25% of patients in groups A, B and C, respectively (P = 0.01). A second dose was administered in 61.5% of patients, with fistular sealing in 39.1%, 43.3% and 37.3%, respectively (P = 0.79). Although the findings were not as promising as in previous studies, posterior analysis stratified by center revealed far better results for patients administered ASCs on comparing the center with the greatest experience *vs* the rest of the participating centers: 45.55%, 83.3% and 18.8% (P = 0.025 for treatment) *vs* 35.8%, 33.3% and 42.6% for groups A, B and C, respectively. The multivariate analysis moreover showed the fact of having received treatment at the center with most experience to be a significant factor. The authors postulated that the different surgical protocol used in the treatment, as well as the experience of the surgeon in using ASCs in perianal fistulas, may have been decisive. As regards the safety of treatment, there were no significant differences in adverse effects among the three groups. A total of 37 serious adverse effects were recorded - three of which were related to the procedures used, but none to use of ASCs.

In that same year, the first study on donor adipose tissue expanded mesenchymal stem cells (eASCs) was published[160]. Up until that time, all treatments had been made with autologous MSCs, except in one case where donor cells were used[161]. This study used an eASC formulation prepared by the company Cellerix (now Tigenix). Twenty-four patients with CD and complex perianal fistulas were enrolled. The primary endpoint was fistular closure in weeks 12 and 24, defined as the absence of suppuration, re-epithelization of the external orifice, and the absence of collections as evidenced by MRI. A starting dose of 20 million cells was injected into the fistula, followed by a second dose of 40 million cells in the event of incomplete closure in week 12. Of the 24 patients included in the study, 16 completed the treatment period and, of these, 69.2% experienced decreased suppuration of at least one of the fistulas, 56.3% showed closure of the treated fistulas, and 30% presented closure of all fistular trajectories. The results were confirmed by MRI. There were two serious adverse effects (fever and perianal abscess), possibly related to the intervention, although both showed full recovery.

At present, a phase Ⅲ study is underway involving eASCs for the treatment of complex fistulas in CD patients. This trial will contribute relevant information and may lead to future marketing of the treatment. A new treatment option is thus under development that may revolutionize the management of perianal fistulizing CD. The safety of this cell product appears to have been confirmed by the different studies published to date. Ten years have passed since publication of the first report, and follow-up studies have been made of some of the published series, without the detection of anomalies[162].

CONCLUSION

At present, treatments with mesenchymal and hematopoietic stem cells offer a potential that requires in-depth investigation. Existing studies are encouraging yet inconclusive. We are at a point of inflexion where these new therapies are seen to afford major curative potential. The coming years will be decisive. The information obtained from ongoing and future clinical trials may lead a revolution in IBD management and its impact upon patients. Undoubtedly, as twenty-first century gastroenterologists, we must expand the scope of our specialty and seek multidisciplinary interaction for the benefit of our patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Inflammatory bowel disease: An increased risk factor for neurologic complications

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Abstract

Only a very few systematic studies have investigated the frequency of neurologic disorders in patients with Crohn's disease (CD) and ulcerative colitis (UC), which are the two main types of inflammatory bowel disease (IBD). Results have been inconsistent and variable, owing to differences in case-finding methods and evaluated outcomes in different studies. The most frequent neurologic manifestations reported in CD and UC populations are cerebrovascular disease (with either arterial or venous events), demyelinating central nervous system disease, and peripheral neuropathy (whether axonal or demyelinating); however, the literature describes numerous nervous system disorders as being associated with IBD. The pathogenesis of nervous system tissue involvement in IBD has yet to be elucidated, although it seems to be related to immune mechanisms or prothrombotic states. The recently-introduced tumor necrosis factor (TNF) inhibitors have proven successful in controlling moderate to severe IBD activity. However, severe neurologic disorders associated with TNF inhibitors have been reported, which therefore raises concerns regarding the effect of anti-TNF-α antibodies on the nervous system. Although neurological involvement associated with IBD is rarely reported, gastroenterologists should be aware of the neurologic manifestations of IBD in order to provide early treatment, which is crucial for preventing major neurologic morbidity.

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Key words: Extraintestinal manifestations; Inflammatory bowel disease; Multiple sclerosis; Neuropathy; Stroke; Tumor necrosis factor inhibitor

Core tip: The neurological manifestations in inflammatory bowel disease (IBD) patients are often unrecognized or underestimated. A detailed revision of the literature about the neurological manifestations in ulcerative colitis and Crohn's disease patients is relevant to the IBD community, especially in the biologics era. Gastroenterologists should be aware of the neurologic manifestations of IBD in order to provide prevention and early treatment, which is crucial for preventing major neurologic morbidity.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of idiopathic inflammatory bowel disease (IBD), and they are clearly distinct pathophysiological entities. UC, the most common form of IBD worldwide, is a disease of the colonic mucosa only; it is less prone to complications and can be cured with colectomy. In contrast, CD is a transmural disease of the gastrointestinal mucosa which can affect the entire gastrointestinal tract

from the mouth to the anu[1,2].

CD and UC should be considered systemic diseases since they are associated with clinical manifestations involving organs outside the alimentary tract. Extraintestinal manifestations (EIMs) involving several organs, and most EIMs occurring in the joints, skin, mouth, eyes and coagulation system, either precede the onset of intestinal manifestations or appear and evolve in parallel with them. They also respond to treatment for the underlying bowel disease. However, many EIMs tend to follow a course independent from that of bowel disease activity. These EIMs are observed in some 20%-40% of patients with IBD, with CD patients being more susceptible to EIMs than patients with UC[3-8].

Neurologic involvement associated with IBD is frequently underreported. Nevertheless, it is important to quantify the morbidity burden of clinically significant neurologic complications in IBD because early recognition and treatment of neurologic diseases are crucial for preventing major morbidity[9,10]. The available literature consists of case reports and small series and only a few of them have reviewed large groups of IBD patients to identify neurologic symptoms. Moreover, most of the recent reviews dealing with UC and CD include only a brief mention of nervous system involvement in IBD[2-5].

The pathogenesis of neurogenic disorders associated with IBD has not been established and it may involve diverse causes. Most of them have an immune basis, but other reported causes include prothrombotic states, nutrient deficiency (vitamin B_{12}, folate, copper, thiamine, vitamin E) because of malabsorption, and iatrogenic complications of medical and surgical management of IBD. In addition, in recent years, use of tumor necrosis factor (TNF) inhibitors has emerged as a successful treatment for refractory IBD, although anti-TNF-α antibodies appear to predispose some patients to developing diverse peripheral and central nervous system (CNS) involvement. An approach to neurologic symptoms in patients diagnosed with IBD has recently been reported elsewhere[11].

Neurologic involvement in IBD as a subgroup of the EIMs may precede the appearance of digestive symptoms or develop after diagnosis of IBD. In addition, neurological symptoms may exacerbate during flare-ups of IBD or evolve independently from intestinal manifestations without responding to treatment provided for the underlying bowel disease[12,13].

This review will examine current knowledge about nervous system involvement in CD and UC and the neurologic manifestations secondary to the use of biological agents and other approaches to IBD management.

EPIDEMIOLOGY OF NEUROLOGICAL MANIFESTATIONS OF IBD

Few systematic studies have investigated the frequency of neurological disorders in patients with IBD. Additionally, results from these studies have been inconsistent, which is mainly due to discrepancies in case-finding methods. In most of the studies reported, magnetic resonance imaging was not part of the standard workup. Moreover, some studies have included neurological symptoms of iatrogenic origin or symptoms caused by malabsorption-related disorders secondary to vitamin deficiencies[14,15]. Most of these studies were retrospective and register-based, and only a few included a control group[16]. Finally, the latest reports on neurologic complications in IBD disease have focused on peripheral nervous system (PNS) involvement, and they lack data on clinical manifestations involving other nervous system components[14,17-20]. Table 1 presents clinical studies addressing the frequency of neurogenic disorders in CD and UC. In a large retrospective register-based study including 638 patients with UC or CD, Lossos *et al*[21] found neurological involvement in 3% of the cases. In contrast, 33.2% of patients in a CD population experienced neurological or neuropsychiatric complications, although this proportion decreased to 19.3% when considering only those cases with a direct relationship[15]. Finally, in another study, 67% of patients with CD and 53% of patients with UC had neurologic disorders, although authors did not report whether IBD and neurologic involvement were coincidental[19]. Table 2 lists neurological disorders reported in the literature as being associated with IBD.

NON-DRUG-INDUCED NEUROLOGICAL MANIFESTATIONS OF IBD

Peripheral neuropathy

Peripheral neuropathy (PN) is known to be related to IBD and it is one of the most frequently reported neurologic complications. Various studies have found PNS complications, rather than CNS involvement, to be predominant[14]. Initially, medical treatments for the gastrointestinal disease or vitamin deficiencies caused by malabsorption were thought to cause PN[21]. Ultimately, PN is a common adverse event associated with use of TNF inhibitors[22]. If these causes of neuropathy are excluded, however, the reported frequency of PN in IBD will vary greatly among published studies, with estimates ranging from 0% to 39% due to selection bias, use of different definitions of the disease, or population characteristics[14,15,18,20,21,23].

Two recent studies have indicated the low prevalence of PN in IBD. In the first study, carried out in Greece, 97 patients with any form of IBD were studied to identify any cases of PN. Fifty-two patients were excluded for different reasons, including presence of comorbidities associated with PN and presence of other neurologic symptoms. Nerve conduction studies in the remaining 45 asymptomatic patients yielded normal results except for one patient with a history of acute motor sensory polyneuropathy complicating UC and another patient with incidental carpal tunnel syndrome[17]. A second study in Olmsted County, Minnesota, determined the neuropathy incidence rate in a population-based cohort of patients

Table 1 Clinical studies of neurologic disorders in inflammatory bowel disease

Author	Year	Country	Study design	Total patients	Type of IBD	Patients with NC	Age (range, yr)	Sex	Other EIM	Associated with IBD exacerbation	Incidence of NC
Lossos *et al*[21]	1995	Israel	Retrospective, computerized search	638	377 CD 261 UC	10 CD patients 9 UC patients	11-71	11 M 8 F	50%	2 patients (10%)	19 patients (3%)
Elsehety *et al*[15]	1997	United States	Retrospective	253	CD	Probable: 49 patients Possible: 35 patients Total: 84 patients	NR	NR	NR	5 patients (6%)	Probable: 19.3% Possible: 13.9% Total: 33.2%
Oliveira *et al*[14]	2008	Brazil	Prospectively, clinic-based, PN especially studied	82	31 CD 51 UC	16 CD patients 23 UC patients	43 CD[1] 51 UC	15 M 24 F	NR	NR	PN: 51.6% CD 45.1% UC Headache: 54.8% CD 57% UC
Sassi *et al*[23]	2009	Tunis	Prospective, prevalence study PN	102	88 CD 14 UC	6 UC patients[2] 3 CD patients	22-64	7 F 2 M	33%	29%	8.80%
Benavente *et al*[12]	2011	Spain	Retrospective, hospitalized patients	84	NR	13 UC patients 12 CD patients	17-74	12 M 13 F	4%	10 patients (40%)	30%
Shen *et al*[20]	2012	United States	Questionnaire-based, study of PN symptoms only	173	102 CD 71 UC	67 patients	NR	NR	NR	NR	38.70%
Figueroa *et al*[18]	2013	United States	Retrospective, observational population based-cohort	772	342 CD 430 UC	9 patients/12 events 6 UC patients 3 CD patients	21-83	5 F 4 M	NR	8 patients	72 patients/100000 person-years
Babali *et al*[17]	2013	Greece	Prospective study of subclinical PN in asymptomatic patients	45[3]	30 CD 15 UC	0 patients	NR	NR	NR	NR	No patients had subclinical neuropathy

[1]Mean age; [2]Three patients had mononeuropathies due to rapid weight loss. Three patients had sensory large-fiber polyneuropathy and two had small-fiber polyneuropathy. One patient had a sensorimotor axonal PN. An etiological factor was identified in four cases (diabetes mellitus and long-term metronidazole treatment); [3]Two patients were excluded: one had history of acute motor sensory polyneuropathy complicating UC and other patient had incidental carpal tunnel syndrome. IBD: Inflammatory bowel disease; NC: Neurologic complications; EIM: Extraintestinal complications; CD: Crohn's disease; UC: Ulcerative colitis; F: Female; M: Male; NR: Not reported; PN: Peripheral neuropathy.

newly diagnosed with IBD over a 64-year period. The overall incidence rate was 72 cases of neuropathy per 100000 IBD person-years with a cumulative incidence rate after 30 years of 2.4%. Moreover, the study found late occurrences of neuropathy in the course of IBD, mainly during IBD inactivity[18].

Regarding the type of neuropathy reported, studies describe demyelinating or axonal involvement of peripheral nerves in IBD, and both neuropathies may be acute or chronic. Several cases of immune-mediated neuropathies in IBD patients have also been reported[24-26]. In a retrospective review of patients with PN and IBD, more than two-thirds of the cases had axonal neuropathy. Neuropathies were predominantly sensory (either small-fiber or large-fiber) rather than sensorimotor for axonal neuropathies; only one-third of patients developed demyelinating forms of neuropathy[19]. Conversely, the clinical spectrum reported by Figueroa *et al*[18] consisted of monophasic immune radiculoplexus neuropathy and chronic distal sensorimotor polyneuropathy. The underlying pathophysiology in cases of neuropathy in IBD patients remains obscure. T-cells are clearly involved in the pathogenesis of demyelinating neuropathies. At the same time, the relationship between axonal damage and immune system disturbances remains unclear, but clinical improvement in patients treated with immunomodulatory agents suggests that there is a link[19].

Cerebrovascular disease

Thromboembolic complications are two to four times more likely in patients with IBD than in healthy individuals; they occur at any age in both sexes and active IBD may increase the relative risk of such complications by as much as 15-fold[27]. The incidence of thromboembolism in IBD ranges between 1% and 7.7% in clinical studies, with postmortem studies reporting rates of 40%[28]. Deep vein thrombosis and pulmonary embolism are the most common entities, but cerebrovascular disorders also oc-

Table 2 Neurologic disorders associated with inflammatory bowel disease reported in the literature

Cerebrovascular disease
Cerebral infarction
Transient brain ischemia
Cerebral venous thrombosis
Demyelinating disease
Multiple sclerosis
Asymptomatic focal white-matter lesions
Myelopathy
Optic neuritis
Inflammatory pseudotumor
Epilepsy
Seizures
Psychosis
Chorea
Major depression
Autonomic nervous system dysfunction
Vasculitis of the central nervous system
Restless legs syndrome
Sleep disruption
Headache
Cranial neuropathies
Melkersson-Rosenthal syndrome
Sensorineural hearing loss
Ischemic optic neuropathy
Bell's palsy
Neuromuscular diseases
Myasthenia gravis
Myopathy
Dermatomyositis
Polymyositis
Vacuolar myopathy
Peripheral neuropathy
Sensory large-fiber polyneuropathy
Small-fiber polyneuropathy
Acute and chronic immune-mediated neuropathies
Monophasic immune radiculoplexus neuropathy
Chronic distal sensorimotor polyneuropathy
Mononeuritis multiplex

cur and they are probably underestimated[29]. In a meta-analysis published in 2013, CD and UC were found to be associated with an increase in the risk of ischemic or hemorrhagic stroke or transient ischemic attack (OR = 1.28, 95%CI: 1.17-1.41), especially among women and young patients[30]. Strokes may affect either arterial or venous territories, but it is unclear whether venous or arterial strokes are more frequent[31,32].

The literature describes cerebral infarction and transient brain ischemia due to small- and large-artery disease involving the anterior and posterior circulation. Carotid arterial thrombosis, retinal branch artery occlusion, carotid thromboembolism, cardiac embolism, and paradoxical embolism are other possible pathogeneses[33-36].

IBD accounts for 1%-6% of the total causes of cerebral venous thrombosis (CVT), and CVT develops without there being a temporal relationship with the course of IBD (Figure 1). There are no appreciable differences between IBD-related CVT and non-IBD related CVT in terms of clinical or radiological characteristics, prognosis, or treatment[37-39].

The reason for the increased rate of thromboembolic

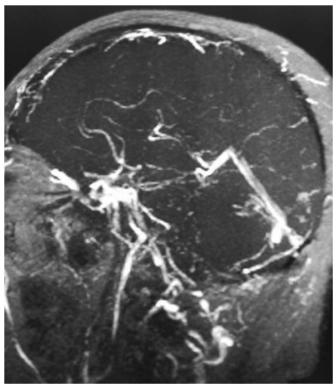

Figure 1 Sagittal magnetic resonance venography imaging scan showing thrombosis of the superior sagittal sinus in a 51-year-old man with cerebral venous thrombosis. He had been diagnosed with ulcerative colitis involving the ileum and colon 15 years before.

events in patients with IBD remains uncertain, but is most likely related to the interaction between acquired and genetic risk factors. Researchers have also reported interaction between cytokine mediators of chronic inflammation and the coagulation cascade[40].

Some genetic factors listed as possible promoters of the thrombotic manifestations of IBD include factor V Leiden and factor Ⅱ, methylenetetrahydrofolate reductase gene mutation, plasminogen activator inhibitor type 1 gene mutation, and factor XIII[28]. However, no studies have convincingly demonstrated that IBD patients have a greater burden of prothrombotic genetic or non-genetic risk factors, such as factor V Leiden mutations, hyperhomocysteinemia, antiphospholipid antibodies, or thrombophilia, than the general population[41-43]. Moreover, genetic risk factors are generally not found more often in IBD patients than in the general population. However, when such factors are present, patients with IBD are more likely than healthy controls to suffer thromboembolic complications[28].

In addition to platelet and endothelial activation, some of the most often-cited impaired coagulation states are high levels of factor Ⅴ, factor Ⅷ, fibrinogen, and von Willebrand factor. In addition, low activity and low concentration of XIII factor subunit A have been detected. This decreased activity inversely correlates with intestinal inflammatory activity and fibrinogen levels[44]. In addition, IBD patients display decreased levels of protein C, protein S, antithrombin Ⅲ, and tissue factor pathway inhibitor (all of which function as anticoagulants), and decreased levels of tissue plasminogen activator with increased plasminogen activator inhibitor-1; the above are all key players in the fibrinolysis pathway[36]. Finally, other mechanisms that may explain cerebrovascular disorders in IBD include vasculitis and consumption coagulopathy leading to hemorrhagic events[21]. Based on these data, recent studies have suggested that IBD may be an independent risk factor for thromboembolic disorders[27,36,45].

Demyelinating disease

The relationship between multiple sclerosis (MS) and

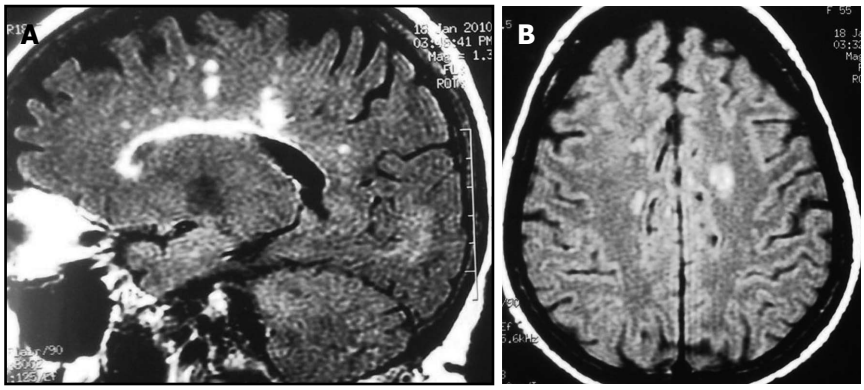

Figure 2 Sagittal (A) and axial (B) cranial magnetic resonance imaging scan depicting several hyperintense focal lesions in supratentorial white matter. The patient was a 45-year-old woman with relapsing-remitting multiple sclerosis and a 23-year history of ulcerative colitis.

IBD was discovered by Rang *et al*[46] in the early 1980s when they detected high MS incidence and prevalence while studying patients who had undergone a colectomy for IBD (Figure 2). Retrospective cross-sectional studies have reported an increased incidence of demyelinating disease[16]; however, incidence of MS was reported to be higher in patients with UC than with CD[47]. In addition, one study found a high prevalence of asymptomatic focal white-matter brain lesions in IBD patients[48] but these data not have been confirmed by more recent studies[49]. Moreover, the incidence of white matter hyperintensities on T2-weighted images was found to be higher in CD patients (72%) than in age-matched controls (34%); however, this population was not completely asymptomatic and 20% of the CD patients were on TNF inhibitors.

Several studies have documented additional autoimmune diseases, including IBD, in patients with MS and their relatives; however, results were disparate due to differences in study design[50,51]. In a Danish study, Nielsen *et al*[52] found that MS patients were at a higher risk for developing UC and other autoimmune diseases, but not CD. This hypothesis is supported by a recent systematic review and meta-analysis. Here, the authors reported a significant relationship between MS and IBD (OR = 1.56, 95%CI: 1.28-1.90), although this association was not found in relatives of MS patients[53].

There may be a significant relationship between MS and IBD. This possibility is supported by the role played by immune mechanisms in the pathogenesis of both disorders and by fact that immune system inhibitors that impair cell-mediated immunity are effective for treating both MS and IBD. Disturbances in functional T-cell subsets as well as in antigen-presenting cells have also been implicated. Studies suggest that aberrant proinflammatory activity in particular may be a common pathway leading to the destruction of target tissue in both diseases[3-5].

Miscellaneous

Studies have reported an association between epilepsy and IBD; however, the possible relationship between epilepsy and IBD has not been completely addressed[12,15,21]. Furthermore, seizures may be symptoms of systemic or brain processes such as hypomagnesemia or cerebral venous thrombosis[54,55]. Vasculitis of the CNS and transverse myelitis associated with Jo-1 antibody syndrome are additional complications that have been described in IBD patients[56-58].

In a prospective multicenter study in patients with CD, incidence of restless legs syndrome (RLS) was 43% and prevalence was 30%. This prevalence rate exceeds that of the general population, and the incidence figure is higher than the incidence of many known EIMs of CD. Furthermore, RLS symptoms occurred during or after the onset of CD symptoms in most patients, suggesting a link between CD and RLS[59]. Recent studies have shown that sleep disorders are more common in IBD patients, and treating sleep disruption with a melatonin supplement has been shown to improve sleep in animal models of immune colitis[60].

Some authors suggest that the autonomic nervous system plays a role in the pathogenesis of IBD and that IBD may be a potential neurologic complication of chronic inflammation. On the other hand, autonomic dysfunctions have been reported in patients with IBD; although earlier reports suggested autonomic hypofunction, later studies also identified autonomic hyperreflexia. Sympathetic autonomic neuropathy is an early systemic feature of CD and one that is independent from disease activity. Moreover, clinically manifest autonomic dysfunction is associated with lower quality of life and more intensive healthcare use in IBD patients[61-63].

Sensorineural hearing loss (SNHL), first described in 1982, is currently a well-recognized EIM of UC[64]. In a study of 38 patients with a history of IBD, 59% had inner ear dysfunction that was probably related to gastrointestinal disease. Of the 19 patients with hearing loss (14 patients had UC and 5 had CD), 16 patients had bilateral SNHL; unfortunately, only one patient experienced clinical improvement following medical treatment[65]. The pathogenesis of SNHL associated with UC is not fully understood, and several immune mechanisms may lead to inner ear dysfunction[66]. Bilateral anterior optic neuritis and ischemic optic neuropathy due to UC and CD have been described[67,68]. Lastly, researchers have reported pe-

ripheral facial palsy and Melkersson-Rosenthal syndrome associated with CD[69].

Furthermore, myasthenia gravis (MG), whether in its ocular or generalized form, has been reported in association with both UC and CD. Intriguingly, two case reports of patients with MG and IBD describe clinical improvement of neuromuscular disease and IBD following different surgical procedures, one after thymectomy and the other following proctocolectomy. These reports emphasize the immunological link between MG and IBD[21,70-74]. In addition to MG, cases of IBD associated with polymyositis or dermatomyositis have also been described[75-77].

DRUG-INDUCED NEUROLOGIC MANIFESTATIONS OF IBD

Tumor necrosis factor inhibitor therapy

TNF inhibitors are currently approved as treatment for IBD, including both CD and UC. The proinflammatory cytokine TNF-α has been identified as playing a pivotal role in the inflammatory cascade that causes chronic intestinal inflammation in IBD. Synthetic anti-TNF-α antibodies have been shown to mitigate this inflammatory process. Infliximab was the first TNF inhibitor successfully used to treat IBD. Other drugs with demonstrated efficacy as IBD treatments include adalimumab and golimumab (humanized monoclonal antibodies), and certolizumab pegol (humanized anti-TNF-α antibody Fab' fragment conjugated with a polyethylene glycol molecule). Conversely, etanercept (non-antibody soluble recombinant TNF receptor-Fc fusion protein) is not an effective treatment for IBD[78].

Since TNF inhibitors were first used in 1996[79], researchers have reported an increased risk of CNS events (new onset or exacerbation of demyelinating events, including optic neuritis and MS); in addition, PNs have also been reported in small clinical series. Information regarding management and clinical significance of such events is very limited because the incidence of neurologic events is estimated at less than 1 event per 1000 patients during TNF-inhibitor treatment[78,80-82].

The FDA's most recent report on adverse events related to TNF-α inhibitors from dates to December 31, 2009. Here, the second-highest number of reported adverse events, after patients with rheumatoid arthritis, was in patients with IBD (140 cases, 18.1% of the total reports). These figures show that IBD may be a risk factor for neurologic disease. Most of the cases involving infliximab and adalimumab are linked to PN (42%), followed by CNS or spinal demyelination (17.2%), optic neuritis (17.4%) and facial palsy (7.8%). In addition, authors reported 19 patients diagnosed with transverse myelitis, leukoencephalopathy, unspecified demyelinating disease, encephalopathy, meningitis due to *Listeria*, or right cerebellar neuroglial cyst. Last of all, one case of PN and another case of CNS demyelination associated with certolizumab (17.2%) have also been reported[22]. These data are consistent with results from the BIOEGAS registry of the Spanish Society of Internal Medicine which reported a total of 175 cases of central demyelinating processes and 44 cases of PNs resulting from TNF-inhibitor therapy as of July 2009[83]. Recent studies have highlighted the development of posterior reversible encephalopathy syndrome in pediatric patients with IBD treated with infliximab[84]. Although several hypotheses have been proposed in an attempt to explain the possible relationship between TNF inhibitor treatment and CNS events, none is considered to be completely satisfactory. Some authors propose that systemic administration of TNF-α inhibitors may enhance different functions of the immune system which are known to activate demyelinating processes[85-87]. However, other authors prefer to seek genetic connections, such as haplotypes of TNF-α or signal transducer and activator of transcription 3 (STAT3), which could explain the emergence of neurologic events in IBD patients treated with TNF inhibitors[88-90].

Regarding PNS disease, most reported types of neuropathy involve demyelination. Cases of Guillain-Barré syndrome account for the majority, but cases of multifocal motor neuropathy with conduction block, chronic inflammatory demyelinating polyradiculoneuropathy, and Lewis-Summer syndrome have also been reported. Moreover, mononeuropathy multiplex and axonal sensorimotor polyneuropathies have been described, which adds an axonal process to the physiopathology of nervous system damage elicited by anti-TNF agents[83,91,92]. The proposed pathogenesis of PNs associated with TNF inhibitors includes a T-cell and humoral immune attack against peripheral nerve myelin, vasculitis-induced nerve ischemia, and inhibition of signaling support for axons[91].

Prognosis in cases of TNF inhibitor-induced neurologic events is usually good if the treatment is discontinued. Gastroenterologists should therefore be mindful of the neurologic complications of biological treatments in order to recognize them immediately; neurological consultations may play a key role in the assessment of these patients[11,22]. In light of the data reported here, use of TNF inhibitors should be avoided in patients diagnosed with PN or MS.

Other biological agents

Natalizumab is an IgG4/κ humanized monoclonal antibody which interferes with the interaction between very late antigen-4, expressed on leukocytes, and vascular adhesion molecule-1, expressed on endothelial cells, thus preventing leukocyte extravasation in inflamed sites[93]. Since natalizumab's mechanism of action differs from that of other biological agents, it represents an important therapeutic option for CD patients who cannot tolerate other treatments or who are experiencing decreased treatment efficacy, particularly when TNF inhibitor treatment does not achieve remission.

The main neurologic adverse event in patients with CD treated with natalizumab is progressive multifocal leukoencephalopathy (PML). The incidence reported in these patients is 2.13 out of 1000 patients. However,

it seems possible to stratify risk of PML among natalizumab-treated patients given that risk mainly tends to be higher in cases of more than 2 years of natalizumab therapy, positivity for anti-JC virus antibodies, and combined use of natalizumab and immunosuppressive drugs[94]. No cases of PML were reported in three recent reports including 154 CD patients on natalizumab[95-97].

Non-biological agents

Cyclosporine is a well-recognized treatment for acute severe UC. Between 10% and 28% of the patients on cyclosporine develop neurotoxicity. The most common associated CNS disorder is a postural tremor that affects the upper extremities and responds to beta-blockers. Severe symptoms affect up to 5% of patients and include psychoses, hallucinations, blindness, seizures, cerebellar ataxia, motor weakness and leukoencephalopathy[98-100].

A pure sensory or autonomic neuropathy is a well-known PNS complication following metronidazole treatment. The neuropathy usually resolves completely once the antibiotic is discontinued[101].

Sulfasalazine (sulphasalazopyrine) is prescribed as treatment for IBD due to its immunomodulatory action. Reported serious adverse reactions include transverse myelitis and encephalopathy[102,103].

Finally, it is well known that psychiatric symptoms can develop in association with administration of corticosteroids in IBD patients, although some authors have reported unusual cases of psychotic episodes in UC and CD patients who were not receiving steroids[104-108].

In conclusion, reports about the incidence and type of neurologic diseases associated with CD and UC are controversial. Therefore, prospective studies are needed to clarify these points, as well as basic studies addressing the multiple mechanisms responsible for nervous system damage.

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TOPIC HIGHLIGHT

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Epidemiological studies of migration and environmental risk factors in the inflammatory bowel diseases

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Abstract

Inflammatory bowel diseases (IBD) are idiopathic chronic diseases of the gastrointestinal tract well known to be associated with both genetic and environmental risk factors. Permissive genotypes may manifest into clinical phenotypes under certain environmental influences and these may be best studied from migratory studies. Exploring differences between first and second generation migrants may further highlight the contribution of environmental factors towards the development of IBD. There are few opportunities that have been offered so far. We aim to review the available migration studies on IBD, evaluate the known environmental factors associated with IBD, and explore modern migration patterns to identify new opportunities and candidate migrant groups in IBD migration research.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Epidemiology; Risk factor; Environment; Hygiene hypothesis

Core tip: Inflammatory bowel diseases (IBD) are well known to involve genetic and environmental risk factors. Cohorts from low IBD prevalence regions migrating to areas of high IBD prevalence are candidates to assess further environmental factors - both protective and promotional. There are few opportunities to identify migratory populations to highlight these effects of environmental risk factors in the development of IBD and to compare first- and second-generation migrants. This review highlights known migratory cohorts and identifies an emerging cohort in Middle Eastern migrants to Australia.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are idiopathic chronic diseases of the gastrointestinal tract well known to be associated with both genetic and environmental risk factors. With increasingly new and powerful genetic techniques, it is worthwhile to re-evaluate the contribution of the environment to the development of both Crohn's disease (CD) and ulcerative colitis (UC). To do this, epidemiological research is the key, and such studies have already identified the increasing global incidence and prevalence of IBD in developing countries as these countries become more developed and "Westernised", highlighting the significance of environmental factors on influencing IBD development globally[1]. However, despite the no-

table amount of studies already conducted on this issue, many of the suggested environmental risk factors for CD and UC still remain contentious in regards to their exact relationship with the diseases[2].

Currently, only smoking has been an established risk factor for CD[3] but paradoxically is protective for UC as found by several studies[4-6]. There seems to be a dose-response relationship between smoking and IBD[7] and childhood exposure to tobacco smoke is associated with a higher risk of developing CD[8]. A meta-analysis found that oral contraceptive pill (OCP) increased the risk of both CD and UC, with a dose-response relationship for CD, and OCP discontinuation reduced IBD risk[9]. Appendectomies are found to have an inverse relationship with UC[10]. For CD, some suggest insignificant association with appendectomies[11,12], while others demonstrate increased risk[12-15]. A meta-analysis found breastfeeding to be protective for both CD and UC[16]. However, this result is not reproducible by several studies[17,18]. Generally, antibiotics are found to be a risk factor for CD[18-20], and a case-control study has found a positive association between CD and UC[21]. Regarding diet, the literature is inconsistent and no clear dietary risk factors for CD and UC can be determined[2]. Furthermore, there are also investigations into "proxy measures" of the hygiene hypothesis, including family size and rural dwelling. The hygiene hypothesis has notable popularity as it fits the observation that incidence and prevalence of autoimmune diseases including IBD increase in nations with improvements in hygiene as a result of modernisation[22]. Improved hygiene may alter intestinal commensal bacteria colonisation, reduce exposure to infectious diseases and vaccinations, which are all speculated to play a role in IBD pathogenesis[23]. However, many studies investigating factors related to hygiene have conflicting results[24]. Overall, the epidemiological studies on IBD environmental risk factors have produced important but inconsistent findings, and methodological alterations in future studies are worthwhile to be considered.

We propose a different method to approach epidemiological research on IBD environmental risk factors: using migrant groups as the study population of epidemiological studies. Groups migrating from areas of low IBD incidence to areas of high IBD incidence will provide insight into the effects of environmental triggers on disease development better than populations without such a transition in environment due to the shortened interval between risk factor exposure and IBD onset. Furthermore, studying the offspring of migrants will provide new cohort data on generational changes and their relationship with IBD development and highlight the contribution of these environmental factors at different ages. We aim to review the available migration studies on IBD and explore modern migration patterns to identify new opportunities in IBD migration research. In particular, we propose that a suitable migrant group for environmental risk factor studies is the migrant population from the Middle East to a developed country such as Australia.

SEARCHING

A comprehensive search on the Ovid MEDLINE database (1946 to present) was performed using the following keywords: "inflammatory bowel disease", "CD", "UC", "epidemiology", "incidence", "prevalence", "clinical characteristics", "extraintestinal manifestations", "risk factors" and "migrant". The search was limited to the English language. A manual search of reference lists of all original articles retrieved was also conducted. For migrant studies, 3 were selected after excluding studies which did not investigate at least two generations within migrant groups. To study potential new migration groups that might provide new IBD research opportunities, we searched for epidemiological studies relating to populations of Middle Eastern heritage and their immigration to Australia. The search terms "Middle East", "Arabic" and "Australia" were used and there were a total of 23 IBD articles pertaining to the Middle East and 2 pertaining to Australia. Figure 1 discloses the literature search, included and excluded articles.

RESEARCH

Advantages of migrant studies

Studies of migrant populations identify disease characteristics of a group in their original location, and trace the group through several generations in the new host country to provide deeper insights into associations between environmental factors and disease expression. Migrant populations have been identified as the most promising model for investigating the changing epidemiology of IBD previously[25]. A criticism of prior risk factor studies is the long time interval between presumed exposure to the environmental trigger and disease development[26], creating uncertainty for etiology. Migrant studies are able to overcome this since migrant cohorts transitioning from developing to developed countries offer a shorter time period between risk factor exposure and disease onset, and many risk factors are postulated to be associated with the lifestyle of developed, Westernised countries. Another advantage is that as immigrants adopt new lifestyles, investigating disease characteristics in successive generations will demonstrate temporal relationships between environmental triggers and development of disease, provided that intermarriage with natives is uncommon[27]. Migrant studies also have the added advantage of overcoming data incompatibility that often occurs when comparing epidemiological data of an ethnic group from a developing country to a similar ethnic group in developed nations due to inequality of diagnostic facilities and healthcare access.

Existing migrant studies

In Leicestershire, United Kingdom, a retrospective cohort study compared the incidence of UC in first and second generation South Asian immigrants to Europeans using both hospital and general practice sources. Stan-

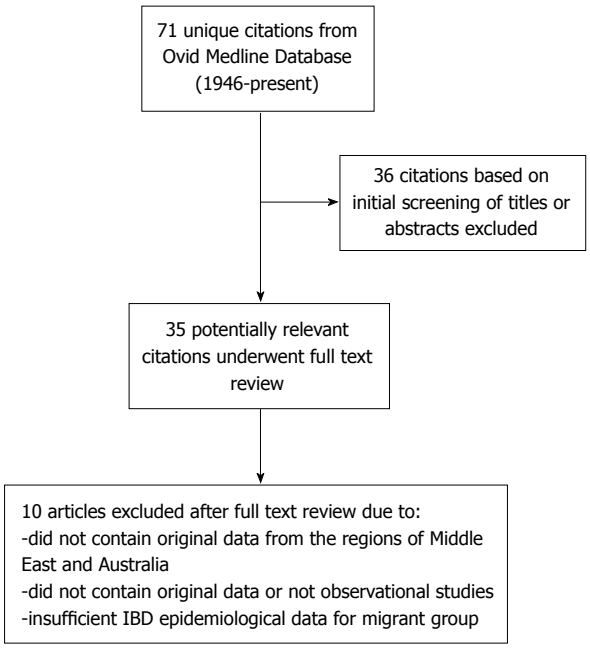

Figure 1 Included and excluded articles. IBD: Inflammatory bowel diseases.

dardised UC incidences for Europeans and South Asian migrants were 5.3 per 10^5 and 10 per 10^5 respectively. Additionally, disease distribution was similar between the two groups, while bimodal age distribution of UC is evident in the European population but not shared by the South Asian migrants. Although unable to accurately determine UC incidence in the two generations separately, the study estimated that first and second generation South Asians are likely at equal risk in developing UC using age-standardised incidences[28,29]. However, a later prospective multicentre study using hospital-based data also conducted in Leicestershire found higher UC incidences for both the Europeans and South Asian first and second generation immigrants (7.0 per 10^5 and 17.2 per 10^5 respectively). Importantly, second generation South Asians were found to suffer higher rates of extensive colitis compared to the first, and were similar to the Europeans[30]. The findings from these two studies challenge earlier Asian epidemiological studies[28,31-42] which reported lower UC incidences, and that environmental impacts cause more severe disease phenotype in later generations. This strongly supports the influential role of environmental risk factors on IBD, which is best investigated by migrant studies comparing successive generations.

Another notable epidemiological study focusing on migrant populations was conducted in Sweden and used a well-defined open cohort study of first and second generation immigrants from 19 countries. The overall IBD risk compared to native-born Swedes was found to be decreased for all first generation immigrants (standard incidence ratios: CD 0.75 per 10^5, UC 0.81 per 10^5). However, when separated into individual countries, several migrant groups originating from developing countries showed increased risk (Figure 2). The highest standardised incidence ratio in second generation immigrants for developing CD were those with one parent or two parents born in Iraq (1.85 and 1.99 respectively), and for UC were immigrants with one (1.55) or both parents (1.68) born in Iran[43]. These results not only suggest that environmental risk factors have varying effect on risk of IBD depending on ethnicity, but also confirm that migrants moving from developing to developed countries may be most susceptible in demonstrating the effects of IBD development due to environmental triggers. Furthermore, the study identified that one of the region which contained the greatest rise of IBD incidence in migrants as the Middle East (Lebanon, Iraq and Iran). Despite the lack of confirmed case ascertainment, this nationwide study is the largest study to date which has investigated generational IBD epidemiological patterns for a large range of migrants from developed and developing countries (Figure 2).

Currently, many studies which compare different ethnic groups within developed and developing countries exist[38,44-48]. These studies are able to characterise ethnic differences in IBD, but for purposes of identifying novel risk factors in IBD expression for particular ethnic groups, migrant studies tracing generational changes are superior since genetic variables are controlled. The studies from Leicestershire and Sweden highlight that generational investigations show highly significant IBD epidemiological differences that are most likely due to environmental causes. Unfortunately, the studies have only investigated incidence and prevalence rates. Future studies should additionally investigate risk factor profiles in successive generations. Furthermore, exploring generational changes are important for understanding the role of family history in IBD expression. A study in Leicestershire found the risk of developing UC was 15-fold for first degree relatives of European patients and 3.5-fold for South Asians. However, prevalence in siblings was comparable between the two ethnicities[48]. Examining generational alterations will shed greater light on the role of inheritance versus environmental contribution towards the development of IBD.

When considering the results of migrant studies, one must be aware of their limitations. Health seeking behaviour and health literacy may be lower in migrant groups, hence reducing IBD detection. If this is the case then any increase in incidence and prevalence reported in migrants in developed countries may be even higher than recorded. Generational environmental changes may also mask the absolute risk of developing CD or UC in immigrants[43]. For example Swedish Iranian immigrants have higher smoking rates than Iranians in Iran[49], which may reduce UC incidence for the immigrants as smoking is protective. These examples would reduce the incidence of IBD in migrants. Therefore results that demonstrate an increase in IBD incidence are likely to be of importance. Finally, it is also important to remember that when interpreting risk factor studies, ethnic factors such as diet may be difficult to differentiate from true environmental influences in epidemiological studies[26]. This may affect

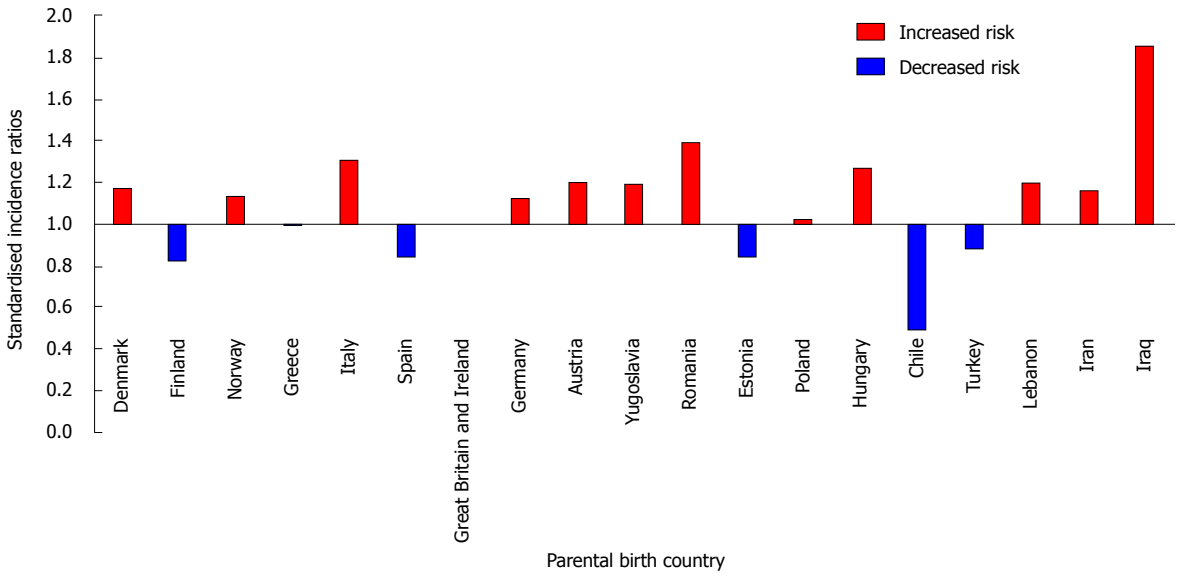

Figure 2 Summary of standardized incidence ratio for Crohn's disease and ulcerative colitis in second-generation immigrants (adapted from ref. [43]).

migrant studies which focus on specific ethnic groups. One way to minimise these confounders is to use controls of the same ethnicity and socio-cultural background.

In the light of the findings of previous migrant studies and modern migration patterns, we aim to identify appropriate migrant groups for future epidemiological research on the risk factors of IBD. Ideally, candidate groups should have good quality epidemiological data on IBD in the country of origin and in the host country, particularly in confirming low IBD incidence rates in the country of origin and high IBD incidence rates in the host country. We propose that migrants from the Middle East migrating to developed countries, such as Australia, are suitable candidates for such studies.

EPIDEMIOLOGY OF IBD IN THE MIDDLE EAST

There is a relatively significant amount of epidemiological research in certain Middle Eastern countries, considering the developing nature of this region. Overall there are 20 incidence[31-34,37,38,47,50-61] and 19 prevalence[31,32,35,36,50,51,53,55,56,58,60,62-65] studies on CD and UC in the Middle East from the countries of Lebanon, Israel, Kuwait, Turkey and Oman. Tables 1 and 2 demonstrate the incidence and prevalence findings of these studies stratified by CD and UC, including references for each specific study. Eleven studies[52-58,64-67] had case ascertainment of UC and CD using the internationally accepted Lennard-Jones criteria, and two were prospective cohort studies[56,59].

Incidence and prevalence

In the Middle East, annual incidence rates for CD range from 1.1 per 10^5 in Beer Sheva[36] to 5.0 per 10^5 in the Kibbutz settlements in Israel[55], while ranges were from 1.35 per 10^5 in Oman[59] to 6.3 per 10^5 in Jerusalem in Israel[37] for UC. The prevalence rates for CD range from 3.2 per 10^5 in the Bedouin Arabic population in Southern Israel[62], and 67.9 per 10^5 in Kibbutz settlements in Israel[64], while ranges were from 4.9 per 10^5 in Turkey[58] to 168.3 per 10^5 in the Kibbutz settlements in Israel for UC[66]. Such data indicate that IBD incidence and prevalence are up to 20-fold lower than other locations in the world. In Europe, North America and Australia the highest UC incidence rates of 24.3 per 10^5, 19.2 per 10^5 and 17.4 per 10^5 have been recorded respectively[68]. UC in the Middle East appears to be of much rarer occurrence. This similarly applies for CD when comparing the Middle East to the world's highest incidence rates.

Despite the relatively rarity of disease in the Middle East homelands, some studies are able to provide temporal trends and demonstrate increasing incidence for both UC and CD. This contrasts with the stabilisation of incidence rates in many established high incidence areas in the world such as Scandinavia[69]. This supports the hypothesis that there are risk factors specific to the western lifestyle that increase IBD incidence. The observation that IBD is more common in European-American born Jews compared to Asian-African born Jews in central Israel[31] is consistent with this. On the other hand, such an increase may be artificial, reflecting increasing physician awareness of IBD and access to diagnostic tools. Additionally, reliability of results is reduced by lack of national population-based registries, compromising incidence and prevalence calculations.

Demographic features

There are 6 studies each[31,32,47,50,52,58] which stratify incidence rates by sex and/or age. The female to male ratio for CD ranged from 0.74 per 10^5 in Tel Aviv Yafo in Israel[31] to 1.43 per 10^5 in Southern Israel[47], and for UC from 0.59 per 10^5 in Trakya Turkey[58] to 0.96 per 10^5 in Tel Aviv Yafo in Israel[32]. Globally, there is a slight female dominance in CD[7,69], but overall sex differences for UC

Table 1 Summary of incidence rates for Crohn's disease and ulcerative colitis in the Middle East stratified by geographical region

Country	Region	Crohn's disease incidence rate (/10^5)	Ulcerative colitis incidence rate (/10^5)	Method	Study period	Ref.
Israel	Upper Galilee		2.33	Survey based study which included all cases in region	1967-1986	Niv *et al*[51], 1990
	Tel Aviv Yafo	1.55		Prospective multicentre study including all hospitals and gastroenterology departments in region	1970-1980	Fireman *et al*[31], 1989
	Tel Aviv Yafo		3.86	Population-based study	1970-1980	Grossman *et al*[32], 1989
	Tel Aviv Yafo		3.66	Survey based multicenter study with well defined catchment area	1961-1970	Gilat *et al*[50], 1974
	Tel Aviv Yafo	1.28		Survey based	1970-1976	Rozen *et al*[33], 1979
	Southern Israel	4.2		Hospital and out-patient based, with complete case ascertainment	1968-1992	Odes *et al*[47], 1994
	Southern Israel		2.98	Retrospective study, hospital and community clinic based	1961-1985	Odes *et al*[34], 1987
	Kinneret Subdistrict		3.5	Population based study	1965-1994	Shapira *et al*[52], 1998
	Kinneret Subdistrict	1.96		Hospital serving a well-defined catchment area covering all IBD cases in district	1960-1990	Shapira *et al*[53], 1994
	Beer Sheva		2.87	Retrospective study, hospital based	1961-1985	Odes *et al*[34], 1987
	Beer Sheva	1.1		Population-based study	1961-1980	Krawiec *et al*[36], 1984
	Beer Sheva	2.1	5.4	Retrospective and prospective cohort study, hospital and community clinic based	1979-1987	Odes *et al*[38], 1989
	Jerusalem		6.3	Survey based	1973-1978	Jacobsohn *et al*[37], 1986
	Kibbutz settlements	5.0		Community-based survey of physicians	1987-1997	Niv *et al*[54], 1999
Lebanon	Kibbutz settlements		5.04	Community-based survey of physicians	1987-1997	Niv *et al*[55], 2000
	National	1.4	4.1	Population based study using a health-maintenance organisation based with large catchment area	2000-2004	Abdul-Baki *et al*[56], 2007
Turkey	National	2.2	4.4	Questionnaire-based multicentre study	2000-2003	Tozun *et al*[57], 2009
Oman	Trakya		0.77	Cross-sectional study, hospital based	1998-2001	Tezel *et al*[58], 2003
	National		1.35	Prospective cohort study, tertiary referral centre based with large catchment area	1987-1994	Radhakrishnan *et al*[59], 1997
Kuwait	National		2.8	Retrospective cohort study, tertiary referral centre based with large catchment area	1985-1999	Al-Shamali *et al*[60], 2003
	National	0.45	2.27	Population-based study	1977-1982	Al-Nakib *et al*[61], 1984

IBD: Inflammatory bowel diseases

and CD are inconsistent[68], suggesting gender-specific risk factors may not exist. In the Middle East, incidence rates were highest in the second decade of life for CD, and third decade for UC. These peak age groups corresponded to global trend of peak age group in 20 to 29 years old range for both UC and CD[68]. Similarly, the unimodal age distribution found by Middle Eastern studies reflects international results[68,69], as only one third of studies demonstrate bimodal age distribution, all from westernised nations[68]. The significance of a second peak is contentious as it may be due to missed diagnosis when the disease first presented earlier in life. On the other hand, it may indicate populations whose disease is more susceptible to environmental factors rather than genetics, and hence for countries in the Middle East where the one peak age group is relatively older, strong environmental influences may be indicated.

Clinical features

Thirteen studies[52-56,58-60,62,67,70-74] from the countries of Lebanon, Kuwait, Iran, Oman, Qatar and Israel characterised intestinal and/or extraintestinal involvement in CD and/or UC in Middle Eastern populations. However, classifications of the site of CD and UC intestinal involvement were not uniform in the Middle Eastern studies, nor were methods of case ascertainment and type of colonoscopy used. The most common intestinal feature reported by all the studies for CD is the terminal ileum, followed by colonic only and ileocolonic. Globally, ileocolitis is the most common[72]. For UC, it is most commonly found as proctitis, followed by left-sided colitis and pancolitis in the Middle East. This is largely similar to the

Table 2 Summary of prevalence rates for Crohn's disease and ulcerative colitis in the Middle East stratified by geographical region

Country	Region	Crohn's disease prevalence rate (/10^5)	Ulcerative colitis prevalence rate (/10^5)	Method	Study period	Ref.
Israel	Upper Galilee		44.58	Survey based study which included all cases in region	1986	Niv *et al*[51], 1990
	Tel Aviv Yafo	13.28		Prospective multicentre study including all hospitals and gastroenterology departments in region	1970-1980	Fireman *et al*[31], 1989
	Tel Aviv Yafo		55.16	Population-based study	1980	Grossman *et al*[32], 1989
	Tel Aviv Yafo		37.4	Survey based multicenter study with well defined catchment area	1970	Gilat *et al*[18], 1974
	Tel Aviv Yafo	12.31		Survey-based	1976	Rozen *et al*[33], 1979
	Southern Israel	50.60		Hospital and out-patient based, with complete case ascertainment	1992	Odes *et al*[47], 1994
	Southern Israel (Arab population)	3.20	9.8	Prospective cohort study, hospital and community clinic based	1990	Odes *et al*[62], 1991
	Kinneret Subdistrict	20.24		Hospital based serving a well-defined catchment area covering all IBD cases in district	1960-1990	Shapira *et al*[53], 1994
	Beer Sheva		70.6	Retrospective study, hospital based	1985	Odes *et al*[34],1987
	Beer Sheva	14		Population-based study	1980	Krawiec *et al*[36], 1984
	Beer Sheva	30	89.0	Retrospective and prospective cohort study, hospital and community clinic based	1987	Odes *et al*[38], 1989
	Kibbutz settlements	65.1		Community-based survey of physicians	1987-1997	Niv *et al*[54], 1999
	Kibbutz settlements		144.1	Community-based survey of physicians	1987-1997	Niv *et al*[55], 2000
	Kibbutz settlements		121.08	Community-based survey of physicians	1987	Niv *et al*[63], 1991
	Kibbutz settlements		168.3	Survey based, complete case ascertainment and large catchment area	1987-2007	Birkenfeld *et al*[66], 2009
	Kibbutz settlements	67.9		Survey based, complete case ascertainment and large catchment area	1987-2007	Zvidi *et al*[64], 2009
Lebanon	National	53.1	106.2	Population based study using a health-maintenance organisation based with large catchment area	2007	Abdul-Baki *et al*[56], 2007
Turkey	Trakya		4.9	Cross-sectional study, hospital based	2002	Tezel *et al*[58], 2003
Kuwait	National		41.7	Retrospective cohort study, tertiary referral centre based with large catchment area	1985-1999	Al-Shamali *et al*[60], 2003

IBD: Inflammatory bowel diseases

extent of UC in other low incidence countries. The most common extraintestinal involvement was rheumatological conditions, a finding reflected worldwide[73].

Risk factors

Ten studies investigated the risk factor profiles in UC and/or CD populations studied[54-59,64,67,71]. However, only one study - an Iranian study with 258 cases[67] - included controls, highlighting an area that would benefit from more case control studies. The most common risk factor studied was smoking, and generally studies showed smoking as less prevalent in UC subjects, and the opposite for CD. This is consistent with global findings. Results for other risk factors are largely inconsistent between Middle Eastern studies.

Racial/ethnic differences

Due to significant differences in IBD incidences within different ethnic groups in Israel, such that IBD incidence was highest among American and European born Jews than those born in Israel, Asia and Africa[56], it is worthwhile for future studies to distinguish IBD characteristics according to ethnicity. Interestingly, it is observed that the prevalence gap between different ethnic groups is disappearing in the Kibbutz settlements, Tel Aviv Yafo and the South and North of Israel[66]. Studies also show that the risks of developing UC for European and American born Jews and for Jews born in Israel are becoming very similar[34]. It can be inferred that as these countries become increasingly westernised, IBD becomes more prevalent. Further studies are necessary to identify the environmental components, ideally done through investigating generational changes when there is migration from developing to developed countries.

EPIDEMIOLOGY OF IBD IN AUSTRALIA

Incidence and prevalence

Wilson *et al*[74] conducted the first and only prospective population based study to determine Australia's IBD inci-

dence over a period of 1 year using the capture-recapture method with good sample size. The crude annual incidence rate for overall IBD, UC and CD were 29.3 per 10^5, 17.4 per 10^5 and 11.2 per 10^5 respectively. This result in the overall IBD incidence rate of 29.6 per 10^5 after age-standardised to the World Health Organisation world standard population[74]. When compared to global IBD incidence, the most recent systematic review of regional epidemiology demonstrates that Australia has higher UC rates than Europe (12.7 per 10^5), Asia and Middle East combined (6.3 per 10^5), and lower when compared to North America. For CD, compared to Europe (12.7 per 10^5) and North America (20.2 per 10^5), Australia's rates are less, while higher than Asia and the Middle East (5.0 per 10^5)[68]. This study is critical in confirming Australia's high IBD incidence which was not previously established; additionally it supports the assumption that Australia shares similar genetic and environmental risk factors with other westernised nations[74].

An earlier smaller study was conducted in the Hunter Valley region of New South Wales on CD patients between 1967 and 1991. A mean incidence of 1.38 per 10^5 with a 3-fold increase in incidence rates between the first and second 10 years was found. The prevalence of these patients was 34 per 10^5. There was a female dominance (1.4:1) and the most common intestinal involvement site was ileocolonic, followed by colonic only, and jejuna[75]. The study's inclusion of only surgically managed CD means there is an underestimation of the total CD incidence in the area.

The short time period of the study conducted by Wilson *et al*[74] is the main limiting factor. Hence in actuality IBD incidence in Australia could be higher. Additionally, further population-based studies conducted over longer periods should stratify results by ethnic subgroups to better represent Australia's IBD epidemiology considering the multiculturalism of this country.

Clinical features and environmental risk factors

Inflammatory bowel disease presentation in Australia is comparable to many Westernised nations. The bimodal age distribution of IBD in Australia, with 20-24 year old age being the peak age group[74], reflects the findings of some Westernised nations[68]. Phenotypically, for CD terminal ileum involvement is most common (49%), followed by colonic only disease (27%) and ileocolonic (24%). For UC, proctosigmoiditis or left sided colitis was most common (48%), followed by isolated proctitis (35%) and pancolitis (17%)[74]. Thus far, there is a lack of epidemiological studies focused on the adult population on environmental risk factors of IBD. A retrospective study on children in the state of Victoria found high prevalence of urban dwelling in cases.

Migrant populations

Multicultural diversity distinguishes Australia. With net overseas migration comprising 53% of Australia's annual population growth[76] it is evident that several generations of migrants populate the country, causing it to be a prime setting to study environmental risk factors of IBD when tracing generational changes. The 2006 census data indicates 193633 people born in the Middle East who were resident in Australia. This accounted for 4.4% of the overseas-born population. Approximately 40% were born in Lebanon, 16.8% were born in Iraq, 15.7% in Turkey, 11.6% in Iran, 4.0% in Israel, 3.6% in Syria and 1.9% in Jordan[77]. Most of the Middle-Eastern - born population reside in Sydney (58.1%). Arrival peaks occurred in 1977 and 1987 coinciding with the Lebanese Civil War. The union of individuals with a common ancestor known as consanguinity is found in the Middle East with a prevalence of 26% in 1988[78]. As IBD has a genetic component it may therefore be more likely to manifest in this migrant group given suitable environmental condition. This migration of Middle Eastern populations to Australia therefore allows for the study of the development of IBD and allows for comparison of first- and second-generation migrants.

CONCLUSION

Migrant studies exploring generational IBD changes and risk factor profiles contribute towards greater understanding of IBD environmental influences due to the advantages they confer. Modern migration patterns offer unique opportunities for these epidemiological studies. Identifying and studying suitable migrant groups, such as Middle Eastern migrants in Australia, is likely to increase our understanding of the environmental triggers of IBD which thus far remain ambiguous entities.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

What is left when anti-tumour necrosis factor therapy in inflammatory bowel diseases fails?

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Abstract

The inflammatory bowel diseases (IBDs) are chronic incurable conditions that primarily present in young patients. Being incurable, the IBDs may be part of the patient's life for many years and these conditions require therapies that will be effective over the long-term. Surgery in Crohn's disease does not cure the disease with endoscopic recurrent in up to 70% of patients 1 year post resection. This means that, the patient will require many years of medications and the goal of the treating physician is to induce and maintain long-term remission without side effects. The development of the anti-tumour necrosis factor alpha (TNFα) agents has been a magnificent clinical advance in IBD, but they are not always effective, with loss of response overtime and, at times, discontinuation is required secondary to side effects. So what options are available if of the anti-TNFα agents can no longer be used? This review aims to provide other options for the physician, to remind them of the older established medications like azathioprine/6-mercaptopurine and methotrexate, the less established medications like mycophenolate mofetil and tacrolimus as well as newer therapeutic options like the anti-integrins, which block the trafficking of leukocytes into the intestinal mucosa. The location of the intestinal inflammation must also be considered, as topical therapeutic agents may also be worthwhile to consider in the long-term management of the more challenging IBD patient. The more options that are available the more likely the patient will be able to have tailored therapy to treat their disease and a better long-term outcome.

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Key words: Inflammatory bowel disease; Immunosuppression; Anti-tumour necrosis factor agents; Anti-integrin; Long-term outcomes

Core tip: Overall the physician must keep an open mind when treating inflammatory bowel disease. These patients have a long-term incurable condition than can significantly impact on all aspects of their life. Surgery does not cure the disease and thus medications may be required for many decades in order to give the patients a decent quality of life. Both the patient and the physician, therefore, need to remember the "oldies but goodies" but also keep the door open to new innovations and novel therapies.

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INTRODUCTION

The chronic inflammatory bowel diseases, Crohn's disease (CD) and ulcerative colitis (UC), are a huge challenge for the treating physician as these are life-long incurable

conditions that frequently present in the 2nd or 3rd decade of life, a stage in the patient's life where education, social integration and personal identity are key aspects being developed. There is no doubt that the anti-tumour necrosis factor alpha (TNFα) medications are efficacious in the management of both conditions[1-8] but they are, however, not a panacea as they are not effective in all patients and even in those in whom a remission is achieved, the effect may be lost over time.

The efficacy of maintenance therapy in CD with the anti-TNFα medication, certolizumab pegol, has been investigated out to 18 mo. This observed that slightly more than 60% of the original 60% of patients who responded to induction therapy continued to respond, which is encouraging[9,10]. This suggests, however, that by 18 mo only 40% of patients are still getting benefit from this medication. This is similar to the findings for adalimumab where 24% of all patients in the CHARM study remained in response at week 26[3] and after 2 years of adalimumab therapy, between 37% and 50% of these patients were in clinical remission[11]. Published data for infliximab out to 54 wk would also appear to be similar[12]. Additional long-term data, out to 4.5 years, suggest that although efficacy for certolizumab in CD is still present[13], the number of patients continuing to benefit falls with time. The problem for both the patient and physician is that the IBDs are life-long conditions and arguably the best medication options for these patients have only a limited subset of patients in whom they will be of long-term benefit.

In addition to a loss of effect over time, like all medications, there are potential side effects to the use of the anti-TNFα agents. As TNF is involved in the immune-mediated response to infection it is not unexpected that anti-TNF medications are associated with an increased risk of serious and opportunistic infections[14,15], including tuberculosis[16], *Pneumocystis jirovecii pneumonia*[17], and various viral, fungal and bacterial infections[18]. Deramatological side effects are also possible with new onset cutaneous eruptions observed in 20% of CD patients treated with infliximab[19], and immune-mediated cutaneous reactions seen in 11% of patients[20]. This risk is also present with the fully humanised antibody, adalimumab[21]. In addition, the potential risks of medication-induced skin cancers and lymphomas need to be considered[22].

Thus these long-term chronic inflammatory diseases, which may be part of a patient's life anywhere from 10 and 70 years, require medications that will be effective over the long-term with minimal side effects. The development of the anti-TNFα agents has been a magnificent clinical advance in this management of these conditions, but what options are available if they lose effect or side effects necessitate cessation of the therapy?

OLD BECOMES NEW AGAIN

Azathioprine and 6-mercaptopurine

An oldie but a goodie. We must never forget about the older medications that have stood the test of time as they are still frequently used and with more innovative thinking may be able to either enhance the effects of the anti-TNFα agents, or be a backstop if, or when, they are no longer of benefit. Through their effects on the synthesis of nucleic acids, the thiopurines reduce intracellular purine metabolism, induce T lymphocyte apoptosis, cause a reduction in the number of circulating B and T lymphocytes[23], decrease immunoglobulin synthesis[24] and reduce the production of interleukin (IL)-2[25] with the desired effect of reducing inflammation.

In many IBD centres, the measurement of thiopurine methyltransferase (TPMT) activity is frequently undertaken as this is the primary determinant of azathioprine (AZA)/6-mercaptopurine (6-MP) metabolism. For patients with moderate enzymic activity (5-12 U/mL), they are likely to achieve 6-thioguanine nucleotides (6-TGN) levels at standard drug dosing (AZA 1.5 mg/kg/6-MP 1.0 mg/kg), while patients with high enzyme activity (usually > 12 U/mL) may require higher doses than normal. TPMT activity, however, does fluctuate, and TPMT enzymic activity can be induced by AZA/6-MP therapy, while 5-aminosalicylates may cause a mild, but reversible, inhibition of TPMT activity.

The measurement of 6-TGN and 6-methylmercaptopurine (6-MMP) levels are now also frequently undertaken as these levels can correlate with the therapeutic response. A 6-TGN level of between 230-400 pmol/8 × 10^8 RBC has been associated with clinical response, although this data needs to be re-examined in a larger patient cohort. Of note is that 6-TGN levels > 400 pmol/8 × 10^8 RBC are often associated with myelosuppression, while 6-MMP levels of > 5700 pmol/8 × 10^8 RBC can be a cause of hepatotoxicity and other AZA/6-MP-induced side effects[26-29].

Of particular note is that the 6-TGN and 6-MMP levels can be used to determine a patient's compliance and may indicate high TMPT activity with shunting of thiopurine metabolism towards the 6-MMP metabolite and away from 6-TGN. If shunting is observed with high 6-MMP and low 6-TGN levels, the addition of allopurinol (100 mg/d) appears to increase the activity of hypoxanthine-guanine phosphoribosyltransferase, which is the first step in the metobolism of the thiopurines to 6-TGN, resulting in increased 6-TGN levels[30-32]. If allopurinol is used then the AZA/6-MP dose must be markedly reduced, generally the author would reduce it to 25% of the original dose until rechecking of the metabolite profile[33].

If there is loss of response to anti-TNFα therapy then the combination of AZA/6-MP with the anti-TNFα agent could also be of benefit. It is now accepted that the combination of the thiopurines with the anti-TNFαs is more effective for the induction and maintenance of steroid-free remission, and mucosal healing in CD than with the use of either drug alone for up to 1 year in patients who are naïve to both agents[34,35]. The evidence for reclaiming a response to anti-TNFα therapy once lost is not clear, but it is a least a viable option for consideration. The evidence of the combined use of

these agent in UC, however, is not as strong as in CD, but as there is a role for AZA/6-MP in mucosal healing, and protection against the development of colorectal cancer the combination of the two agents would again seem to be reasonable to consider[36].

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is a immunosuppressing agent with similar anti-metabolite and pharmacodynamic properties to the thiopurines, which has primarily been used for preventing the rejection of solid organ transplantants. Its role as an immunosuppresant in the management of IBD has to date been fairly limited with several open labelled studies[37-39] and only a few randomized trials that have been limited by low patient numbers[40-42]. Consideration of its use in the management of difficult IBD cases, however, should not be ignored.

Most early studies investigating the use of MMF were undertaken in CD patients who had failed, or were intolerant to, AZA, and these demonstrated good efficacy[40,42,43]. Unfortunately, these findings were not always reproduced by later studies. These later studies suggested that there was both a low initial response rate as well as a high relapse rate. It was also noted that there was frequently a high medication discontinuation rate secondary to side effects[37,38,41,44,45]. Additional studies comparing the efficacy of AZA to MMF, however, observed that MMF could be more effective in AZA intolerant, rather than refractory patients, while being non-inferior to AZA in the management of UC, for the induction and maintenance of remission at 6 mo[40,46,47]. A longer-term study in a cohort of AZA resistant/intolerant patients, however, observed that although MMF was initially effective, the relapse rates were high, with the suggestion that MMF may be potentially effective but not a long-term solution[48]. There has also been suggested that the MMF dose needs to be increased over time in order to maintain an effect. This has not been the experience of the author as our data demonstrate that MMF was efficacious and well tolerated in treating refractory IBD who are intolerant to AZA/6-MP without the problems of an early disease flare, or the need for dose escalation over time[39].

As many of the studies suggest that MMF is potentially as effective as conventional immunosuppressants when these medications fail, or cannot be used due to hypersensitivity reactions including pancreatitis, then it is a potential alternative that is worthwhile for consideration[46,47]. Further evaluation of its role needs to be undertaken in larger randomized, double-blind studies comparing it to conventional immunosuppressants, however, such studies are expensive and not easy to undertake.

Methotrexate

Methotrexate (MTX) exerts its activity at the DNA level. It inhibits the conversion of dihydrofolic acid to folinic acid, its active metabolite, through the competitive inhibition of dihydrofolate reductase. As folonic acid is required for purine and pyrimidine metabolism and amino acid synthesis, MTX alters their incorporation in the DNA and reduces cellular proliferation, increases T cell apoptosis and endogenous adenosine with alteration of the expression of cellular adhesion molecules and the production of proinflammatory cytokines. The resultant effect is a reduction on systemic inflammation.

Unfortunately, there have been only limited studies investigating MTX in IBD. The largest trial investigated the use of 25 mg/wk intramuscular (*im*), or placebo, in combination with 20 mg/d prednisolone[49]. At 16 wk, significantly more patients receiving MTX were in remission off steroids compared to placebo ($P = 0.025$), however, adverse events were significantly more common with MTX. Two other small trials examining oral MTX 15 mg/wk[50] for 3 mo compared to placebo and oral MTX 12.5 mg/wk in combination with 50 mg/d 6-MP or placebo for 9 mo[51] demonstrated no significant differences between the groups. The second study, however, used suboptimal doses of the immunomodulators, did not have well defined steroid reduction protocols and included patients with known thiopurine-resistant disease.

The use of MTX has been further examined in two open-label studies in CD, the first compared 25 mg/wk MTX intravenously for 3 mo followed by 25 mg/wk MTX orally, with 2 mg/kg per day oral AZA[52] for 6 mo. At 3 and 6 mo there was no difference between the percentage of patients in remission between the MTX and AZA groups, but there were significantly more adverse events with MTX. The second study examined patients naïve to immunomodulator therapy[53] and compared MTX 15 mg/wk orally with 6-MP 1.5 mg/kg per day and 5-ASA 3 g/d for 30 wk. Remission was achieved in 80% of patients on MTX and 94% on 6-MP, but this was not statistically different.

The combination of MTX and the anti-TNF medications has been suggested to be beneficial in paediatric patients with one retrospective analysis[54], and the findings are similar to those seen with the combination of thiopurines and an anti-TNF agent in CD. Expert opinion is also that the combination MTX and an anti-TNF agent can be of benefit in the adult IBD population[55], particularly when the anti-TNF therapy is used episodically. In addition, although the data on the effect that MTX has on mucosal healing is very limited with only a single case series of in CD patients, it does suggest that MTX does have the potential for mucosal healing[56].

Despite the limited number of studies of MTX in the induction and maintenance of remission in CD the conclusion of the Cochran review was that MTX was useful in steroid dependent CD and should be commenced at 25 mg/wk *im* of subcutaneous (SC) and continued for 16 wk[57-59]. Maintenance of remission could then be continued with MTX at 15 mg/wk *im* or SC but with no evidence to recommend the use of oral MTX[60]. The evidence for MTX in UC is even more limited with a single retrospective case series suggesting some benefit, and only a single prospective randomised trial[51]. The efficacy in UC thus appears to be primarily based on anecdotal

experience alone and this is reflected in the Cochran review which stated that there was no evidence for MTX treatment in UC[61-63].

Tacrolimus

Tacrolimus is a macrolide immunosuppressant that is frequently used to prevent the rejection of renal and hepatic allografts. It has the ability to inhibit T cell activation through the formation of an intracellular complex with immunophilins[64] and these bind to, and inhibit, calcineurin, an enzyme involved in the regulation of transcription factors. Tacrolimus thus does not exert its effect through the inhibition of DNA synthesis but instead inhibits T lymphocyte proliferation through the inhibition of proliferative cytokine production like IL-2[65].

Its role in the management of IBD has been investigated in a number of studies, but unfortunately the majority of these are small, retrospective and uncontrolled[66-78]. In general there would appear to be some efficacy with remission rates ranging from 7%-69% in patients with luminal CD[79] and 9%-74%[70,80] in those with UC. The durability of the clinical response, however, is something that may not be optimal[72] with significant variability in the findings and this, in combination with the potential adverse effects of headache, tremor, parasaethesia, insomnia, gastrointestinal upset, arthralgia and particularly nephrotoxity[73,81], makes the use of tacrolimus in IBD somewhat controversial and its use is not wide spread.

Tacrolimus was initially used in the management of perianal CD where for 10 wk patients received tacrolimus 0.1 mg/kg per day or placebo[82]. There was a significant reduction in fistulae drainage in the tacrolimus-treated group of 43% compared to the placebo group of 8% (P = 0.01), however there was no difference in the remission rates between the groups.

The rest of the data for luminal CD comes primarily from retrospective case series that focus on patients who have failed, or are intolerant, to AZA/6-MP. There are also studies that have included patients failing anti-TNFα therapy, but the proportion of these patients is generally low and are in the range from 13% to 47%[67,70,83]. These studies, however, do suggest some efficacy in both the induction and maintenance of remission[68,70,72] with most patients commencing on 0.1 mg/kg tacrolimus twice a day with the aim to get the tacrolimus trough level within the therapeutic range of 5-20 ng/L. Better response and remission rates would appear to be associated trough levels of > 10 ng/L, and these can reach a response rate of between 68% to 83%[73] and a remission rate of 64% in CD patients[72].

In UC patients hospitalised with moderate/severe steroid refractory disease[81], not on AZA/6-MP, the use of tacrolimus after two weeks was associated with a clinical improvement that was statistically significant, and dose dependent, suggesting that high serum concentrations (10-15 ng/L) are more efficacious that low concentrations (5-10 ng/L) or placebo. Tacrolimus was also noted to achieved mucosal healing in 78.9% (15/19) of patients with the high trough levels compared to 44.4% (8/18) of patients with low trough levels and placebo 12.5% (2/16)[81]. The most recent Cochran review[84] also concluded that tacrolimus was effective in inducing a clinical improvement in a dose-dependent manner in treatment-resistant UC with the number needed to treat being 3.

More recently the long-term efficacy of tacrolimus in both CD and UC patients who had failed standard immunosuppressive and anti-TNFα therapy was assessed in a retrospective study with a trough level targeted between 8-12 ng/mL[85]. Clinical response, remission and surgery were then assessed at 30-d, 90-d and 1-year. This paper identified that 65.7% of patients had a clinical response at 30 d, 60% at 90 d and 31.4% at 1 year while 40% of patients were in remission at 30 d, 37.1% at 90 d and 22.9% at 1 year. The risk of surgery was significantly reduced in patients who achieved and maintain a clinical response at 90-d (P = 0.004). The risk of surgery at 1 year was still very high at 40% and almost 60% by 2 years, but the figures were similar to the 50% three year colectomy rate observed in steroid refractory UC patients treated with infliximab as rescue therapy[86]. The findings thus suggest that tacrolimus could induce both a clinical response and clinical remission in medically refractory IBD patients with long-term benefits.

TOPICAL THERAPIES

In all conditions the disease location, severity, patient preferences and allergies need to be considered when prescribing any treatment. Topical tacrolimus has been effective in the treatment of both the perioral and perineal inflammation present in paediatric CD, with resolution of symptoms in up to 75% of patient[87]. More aggressive or novel topical therapy may also be of benefit in distal UC[88,89]. Distal ulcerative colitis (DC), also known as left-sided colitis or E2 disease under the Montreal classification[90], is disease confined to the colon distal to the splenic flexure, while proctitis, or E1 disease (Montreal classification), is disease localized to the rectum. These occur in over 50% of UC patients and, although these result in distressing symptoms, including increased stool frequency, tenesmus, urgency and bleeding, they can often be managed within the community. Resistant disease, however, can be extremely difficult to manage and when there is failure of disease control with routine topical 5-aminosalicylic acid (5-ASA) and steroid therapy, oral agents including the 5ASAs, AZA/6-MP, steroids or an anti-TNFα medication may be use. Unfortunately they do not always help and clinical remission with anti-TNFα therapy only occurs in at most a third of patients[7,91].

It is in these patients that the investigation of other agents is required. To date there have been numerous topical agents proposed for left-sided disease and these have been investigated primarily by open-labelled studies including tacrolimus suppositories[92,93], as well as tacrolimus[93], ecabet sodium[94,95], acetarsol[96] and thromboxane

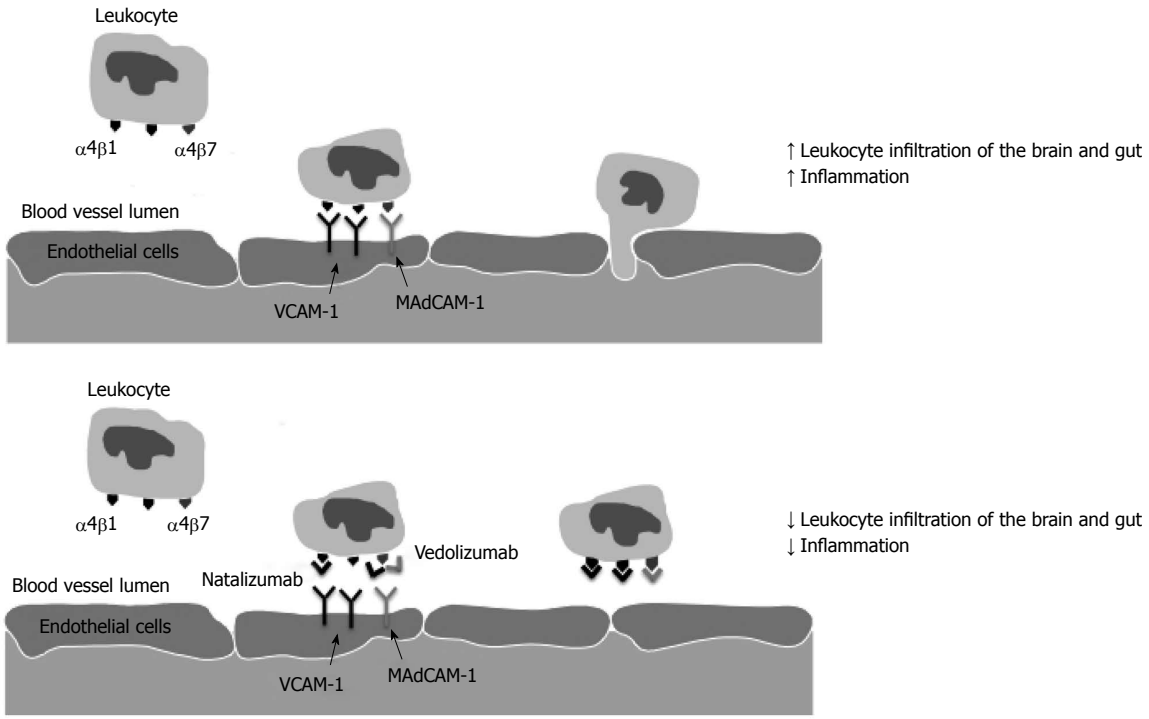

Figure 1 Modification of Leukocyte trafficking by the anti-integrins. VCAM-1: Vascular cell adhesion molecule-1; MAdCAM-1: Mucosal addressin cell adhesion molecule 1.

enemas[97]. Unfortunately none of these have undergone blinded randomised studies as yet, although tacrolimus suppositories are currently being investigated in a double-blind placebo-controlled trial. There are, fortunately, several other agents that have undergone randomised studies, and these include butyrate[98-100], cyclosporine[101] and nicotine enemas[102], however, none have demonstrated better efficacy than placebo in left-sided disease or proctitis. In addition, despite impressive evidence for epidermal growth factor enemas in one small randomized study, the finding has never been reexamined or reproduced[103]. It does appear, however, that the mucosal medication concentration and/or contact time may be important for the topical agents to work[104]. This suggests that enemas are not the best method of administration for patients with proctitis. Further investigation is still required, however, before any of these agents can be considered as routine in the management of DC or proctitis, but the need is great and hopefully further work will be undertaken.

MODIFYING T CELL TRAFFICKING

As inflammation in IBD is thought to result from inappropriate activation of the mucosal immune system by intestinal luminal antigens in genetically susceptible individuals[20,105], the trafficking of leukocytes into the intestinal mucosa would appear to be central to the induction, and maintenance, of the intestinal inflammation in IBD. Trafficking of leukocytes is mediated *via* the recognition of specific adhesion molecules, or integrins that are heterodimeric glycoproteins located on the cellular membrane[106]. These transmembrane receptors consist of an α- and β-subunit with at least 24 different combinations already identified allowing for a wide range of receptor specificity[107]. The α subunit determines the specificity of the interaction between the leukocyte and the endothelial cell and the α4 integrin is widely expressed in both the intestine and brain, and is able to form two different heterodimers with either the β1 or β7-subunit (Figure 1)[107].

The α4β1 integrin is primarily expressed on lymphocytes and monocytes[108], and binds with vascular cell adhesion molecule-1 that is located on vascular endothelial cells allowing cellular migration into the tissue matrix of the brain[109]. The α4β7 integrin demonstrates some overlapping specificity with the α4β1, but also recognises mucosal addressin cell adhesion molecule-1 (MAdCAM-1) that is important in trafficking of lymphocytes into the gut[109,110]. Of particular note is that MAdCAM-1 expression levels are know to be upregulated in association with chronic inflammation in both the small and large intestine of patients with both CD and UC[111,112] and that the α4β7 heterodimer is highly expressed on memory T cells within the intestine[113].

In addition to the integrins there are other proteins that are found on the cell surface of circulating lymphocytes. One of these is chemokine receptor 9 (CCR9) and it is the only known ligand for CCL25, which is expressed by gastrointestinal tract epithelial cells[114]. When CCR9 is expressed on circulating lymphocytes these cells are able to traffic to the intestine[115,116] and thus CCR9 has been implicated in the development and maintenance of the inflammation observed in IBD[117]. Thus modifying the trafficking of these cells may also impact on the development and progression of IBD inflammation.

Natalizumab

The first of the medications to test the concept of altering leukocyte trafficking was Natalizumab (Tysabri, Elan Pharmaceuticals and Biogen Idec), which is a humanised anti-integrin to IgG_4 monoclonal antibody that bound to, and inhibited that binding of the α4 integrin to its target proteins in the brain and the gut and it was shown to be effective in the treatment of multiple sclerosis[118,119]. In the 12-wk induction trial in moderate to severe CD patients, patients were randomly assigned in a 4:1 ratio to receive Natalizumab or placebo with the primary endpoint at week 10 and this was defined as a clinical response with a drop in the CD activity index (CDAI) of ⩾ 70 points[120]. Although the primary end point was not met ($P = 0.05$) post hoc analysis identified that if a CDAI drop of > 100 was used or if patients were stratified for an elevated C-reactive protein at baseline, significance was detected between the groups. In the maintenance study, Natalizumab, however, demonstrated an ability to maintain a clinical response ($P < 0.001$) and remission ($P = 0.003$) compared to placebo. Unfortunately the emergence of the life threatening side effect, progressive multifocal leukoencephalopathy (PML), was associated with the use of Natalizumab and due to this the FDA added the criteria that Natalizumab must not be used in combination with immunosuppressants or inhibitors of TNF-α, and the use of Natalizumab for the management of CD has never been approved in many countries.

Vedolizumab

Natalizumab demonstrated that altering lymphocyte trafficking could effect site-specific inflammation[121,122]. But as this anti-α4 monoclonal antibody targeted both the α4β1 and α4β7, and was associated with an increased risk of PML, the potential of targeting the β7 subunit, or both the α4 and β7 subunits was considered. This would improve antibody specificity by only affecting those leukocytes homing to the intestine, and potentially would have less systemic side effects.

The humanised monoclonal antibody Vedolizumab (Millennium: The Takeda Oncology Company, Cambridge, MA, United States) was developed as a highly selective adhesion molecule antagonist that blocked the interaction between the α4β7 integrin and its ligand thus preventing lymphocyte migration into the gut[123]. Recently the phase Ⅲ induction and maintenance studies for both CD and UC have been presented with very encouraging results. In UC there were response rates at 6 wk of 47.1% in the treatment arm [300 mg intravenously (*iv*) at weeks 0 and 2] compared to 25.5% of patients receiving placebo ($P < 0.001$), while maintenance therapy with iv Vedolizumab at either 4 of 8 weekly was compared to placebo and the percentage of patients who were in clinical remission at week 52 was 41.8, 44.8 and 15.9 respectively ($P < 0.001$ both treatment arms to placebo).

The use of Vedolizumab in CD has also been encouraging. The induction phase was the same as for the UC study and at week 6, 31.4% of patients on Vedolizumab had a clinical response compared to 25.7% of patients on placebo ($P = 0.23$) but 14.5% of patients on active treatment were in remission compare to 6.8% receiving placebo ($P = 0.02$). At week 52, however, the percentage of patients who were in clinical remission was 39.0% (4 weekly infusion), 36.4% (8 weekly infusion) compared to 21.6% receiving placebo ($P < 0.0001$ and $P = 0.004$ respectively). There was noted to be a higher risk of adverse events for patients receiving Vedolizumab, but there were no cases of PML, compared to those getting placebo suggesting that further experience and data collection will be required.

Vercirnon

Vercirnon (CCX282-B) is a selective antagonist of CCR9 with the advantage of being orally active[124] that was initially synthesised by ChemoCentryx Inc, but was subsequently investigated by GlaxoSmithKline where it has just completed the pivotal induction study and was to continue investigation in the SHEID studies for the management of CD. The preclinical studies demonstrated that this molecule inhibits the CCL25-induced chemotaxis[125] and in animal models of colitis, was shown to reduce the severity of intestinal inflammation in the $TNF^{\Delta ARE}$ murine model of colitis[125].

In the two Phase Ⅱ/Ⅲ parallel studies, Vercirnon was examined in moderate to severe active CD. The percentage of patients achieved a clinical response (CDAI decrease ⩾ 70 points from baseline), or remission (CDAI < 150), at 12 wk CD was 61% and 29.9% compared to those getting a placebo of 47.2% ($P = 0.039$) 27.1% (not significant) respectively. The percentage of patients in remission at 52 wk was 47% in the treatment arm and 31% with placebo ($P = 0.012$) suggesting that there was potentially some efficacy of the medication.

Further studies, however, have been unimpressive with the SHIELD-1 study undertaken by GSK determining that in adult patients with moderately-to-severely active CD, Vercirnon did not achieve the primary endpoint of improvement in clinical response nor the key secondary endpoint of clinical remission. Of note was that although the rates of serious adverse events, and withdrawals due to adverse events, were similar among the groups, there was a trend for a dose-dependent increase in overall adverse event rates with Vercirnon. Consequently, GSK has cease all clinical trials into the use of Vercirnon in management of CD until there have been further analysis of the SHIELD-1 findings.

CONCLUSION

There is no doubt that the anti-TNFα medications have been a great addition to the treatment options for both UC and CD with many promoting at "top down" therapeutic approach that commences with an anti-TNFα medication in the management of CD, or a rapid "step up" approach when this is not feasible. These medications, however, do not always induce remission and loss

of response over time, or the development of side effects, may also limit their long-term efficacy.

In all cases the location and severity of the intestinal inflammation with determine which medications are required and the best mode of administration. For the left sided and distal colidities, and perianal CD, topical agents may be the best choice. Particular thought should be put into this by the physician as these often have less systemic side effects than other agents and can be every effective. Unfortunately further investigation is required before many of these will be part of routine management.

The thiopurines have demonstrated long-term efficacy and by measurement of their metabolities and modification of their activity of hypoxanthine-guanine phosphoribosyltransferase with allopurinol, their efficacy may be increased. Use in combination with the anti-TNFα medications is also of benefit and should be considered in all patients in order to prolong and improve the long-term outcomes with these medications. Methotrexate may also be able to be used in a similar manner to the thiopurines and improved patients outcomes. Less recognized are MMF and tacrolimus as medications for use in IBD but these should also be considered when conventional therapies fail or patient intolerances limit the use of conventional therapies.

There are now newer therapies that have been developed to target leukocyte trafficking to the intestine and these have, fortunately, demonstrated clinical efficacy. The most recent is Vedolizumab, which blocks the α4β7 integrin and it achieved demonstrated impressive efficacy for the induction and maintenance of remission in UC and also has a long-term effect on the maintenance of remission in CD. It would thus be expected that very soon this medication will be under consideration by the various regulatory authorities around the world for use in the IBDs thus allow a further therapeutic option.

Overall the physician must keep an open mind when treating IBD. These patients have a long-term incurable condition than can significantly impact on all aspects of their life. Surgery does not cure the disease and thus medications may be required for many decades in order to give the patients a decent quality of life. Both the patient and the physician, therefore, need to remember the "oldies but goodies" but also keep the door open to new innovations and novel therapies.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Campylobacter concisus and inflammatory bowel disease

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Abstract

Investigation of the possible role of *Campylobacter concisus* (*C. concisus*) in inflammatory bowel disease (IBD) is an emerging research area. Despite the association found between *C. concisus* and IBD, it has been difficult to explain how *C. concisus*, a bacterium that is commonly present in the human oral cavity, may contribute to the development of enteric diseases. The evidence presented in this review shows that some *C. concisus* strains in the oral cavity acquired zonula occludens toxin (*zot*) gene from a virus (prophage) and that *C. concisus* Zot shares conserved motifs with both *Vibrio cholerae* Zot receptor binding domain and human zonulin receptor binding domain. Both *Vibrio cholerae* Zot and human zonulin are known to increase intestinal permeability by affecting the tight junctions. Increased intestinal permeability is a feature of IBD. Based on these data, we propose that a primary barrier function defect caused by *C. concisus* Zot is a mechanism by which *zot*-positive *C. concisus* strains may trigger the onset and relapse of IBD.

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Key words: *Campylobacter concisus*; Inflammatory bowel disease; Zonula occludens toxin; Tight junctions; Intestinal permeability

Core tip: *Campylobacter concisus* (*C. concisus*) is an oral bacterium that was previously shown to be associated with inflammatory bowel disease (IBD). Evidence presented in this review shows that some strains of *C. concisus* acquired zonula occludens toxin (*zot*) gene from a virus (prophage), suggesting that a primary barrier function defect caused by *C. concisus* Zot is a mechanism by which *zot*-positive *C. concisus* strains may trigger the onset and relapse of IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract[1]. The two major clinical forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). The etiology of IBD is not fully understood. Multiple contributors including genetic

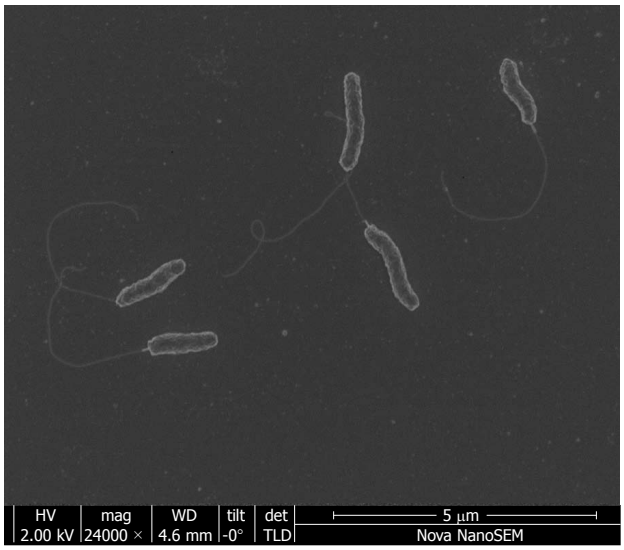

Figure 1 Electron microscopic image of *Campylobacter concisus*.

factors, environmental factors and intestinal microbiota have been suggested to play a role in the development of IBD[1]. The pathogenesis of IBD is thought to result from a dysregulated response of the intestinal mucosal immune system to luminal commensal microbes[1].

The highest incidence of IBD, both CD and UC, is in young adults[2]. This implies that in most of the patients with IBD, the intestinal mucosal immune system has maintained a non-hostile relationship with the intestinal commensal microbes for decades prior to the onset of the disease. Given this, the uncontrolled attack of the intestinal mucosal immune system to luminal commensal bacteria would have been initiated by a trigger. Such a trigger may not necessarily be a dominant intestinal bacterial species or a long-term intestinal resident microbe. Evidence presented in this review suggests that zonula occludens toxin gene (*zot*)-positive *Campylobacter concisus* (*C. concisus*) strains, a bacterium colonizing the human oral cavity, may be a trigger of IBD.

C. concisus

C. concisus is a Gram negative bacterium that requires microaerobic or anaerobic conditions enriched with H_2 for growth[3]. Cells of *C. concisus* are curved, with a size of (0.5 - 1) × (2 - 6) μm. *C. concisus* is motile, driven by a single polarized flagellum (Figure 1).

HUMANS ARE THE NATURAL HOST OF *C. CONCISUS* AND THE ORAL CAVITY IS THE PRIMARY COLONIZATION SITE

Tanner *et al*[4] first reported the isolation of *C. concisus* from patients with gingivitis in 1981. Zhang *et al*[5] examined the presence of *C. concisus* in saliva samples obtained from healthy individuals of different age groups and found that *C. concisus* is commonly present in the human oral cavity. In that study, *C. concisus* was detected in 97% (57/59) of saliva samples collected from healthy individuals aged 3-60 years old by polymerase chain reaction (PCR) targeting 16S rRNA gene and cultured from 75% (44/59) of these saliva samples using a filtration method. A study from Petersen *et al*[6] also detected a high prevalence of *C. concisus* in the human oral cavity: in this study *C. concisus* was detected in 100% of saliva samples (11/11) collected from healthy individuals by a PCR targeting 16S rRNA gene. Despite its high prevalence in the human oral cavity, *C. concisus* is not a dominant oral bacterial species[7].

Table 1 Detection of *Campylobacter concisus* in samples obtained from healthy individuals

Samples	Cultivation of *C. concisus*	Detection of *C. concisus* DNA by PCR
Human saliva	75%	97%-100%
Human feces	0%-2.8%	33%
Human intestinal biopsies	0%	2%-38%

Data shown in this table were obtained from references 5-10 and 17-21. To date, no studies have detected *Campylobacter concisus* (*C. concisus*) in samples obtained from healthy animals. The collective data suggest that humans are the natural host of *C. concisus* and the oral cavity is the primary colonization site. PCR: Polymerase chain reaction.

In comparison to the high isolation of *C. concisus* from saliva samples, the isolation rates of *C. concisus* from fecal samples collected from healthy individuals were much lower. Using the filtration method, Engberg *et al*[8] isolated *C. concisus* from 2.8% (3/107) of fecal samples and Nielsen *et al*[9] did not isolate *C. concisus* from any of 108 fecal samples collected from healthy individuals. The low isolation rates of *C. concisus* from fecal samples suggest that the human intestinal tract of healthy individuals is a less optimal site for *C. concisus* colonization compared to the oral cavity.

To date, *C. concisus* has not been detected in any healthy animals. In a study examining the presence of *Campylobacter* species in fecal samples collected from 70 healthy pet dogs using a quantitative PCR targeting the 60 kDa chaperonin gene, Chaban *et al*[10] detected seven different campylobacter species but not *C. concisus*. Various campylobacter species have been detected in fecal samples collected from animals or birds, but *C. concisus* was not detected[3,10,11]. Lynch *et al*[12] isolated *C. concisus* from 10% (18/185) of chicken meat and 3% of beef meat (6/186) samples. However, whether chicken and cattle are natural hosts of *C. concisus* cannot be determined from these data.

C. concisus has been detected in some animals with gastrointestinal disorders. Petersen *et al*[6] detected *C. concisus* in 12.5% (1/8) of saliva samples from pet cats with dental diseases by PCR targeting the 16S rRNA gene[6]. In addition, Chaban *et al*[10] detected *C. concisus* in fecal samples of 9% of dogs with diarrhea (6/65).

The collective data suggest that humans are the natural host of *C. concisus,* with the human oral cavity being the primary colonization site (Table 1).

Table 2 Genetic relatedness of enteric and oral *Campylobacter concisus* strains

Enteric *C. concisus* strains from patients with IBD	Genetically related *C. concisus* strains
A strain isolated from intestinal biopsies of patient No. 1	An oral strain from patient No. 6
A strain isolated from intestinal biopsies of patient No. 3	An oral strain from patient No. 3 (identical)
A strain isolated from luminal fluid of patient No. 3	An oral strain from healthy No. 1

Data shown in this table were obtained from reference 13. The genetic relatedness was assessed based on the sequences of six housekeeping genes. These data provide evidence showing that *Campylobacter concisus* (*C. concisus*) strains colonizing the intestinal tract of patients with IBD originating from either the patient's own oral *C. concisus* strains or oral *C. concisus* strains from other individuals. IBD: Inflammatory bowel disease.

DIVERSITY OF *C. CONCISUS* STRAINS COLONIZING THE HUMAN ORAL CAVITY

C. concisus strains colonizing the human oral cavity are greatly diverse. On examination of oral *C. concisus* strains isolated from individual patients with IBD and healthy controls, it was found that *C. concisus* strains isolated from each individual had unique protein patterns on sodium dodecyl sulphate polyacrylamide gel electrophoresis[5,13,14]. Furthermore, some individuals were colonized with multiple *C. concisus* strains in the oral cavity, with as many as three different *C. concisus* strains having been isolated from individual patients with IBD or healthy controls[5,13,14]. A significantly higher number of patients with active IBD were colonized with multiple *C. concisus* strains in the oral cavity compared to healthy controls[14].

COLONIZATION OF ORAL *C. CONCISUS* STRAINS IN THE INTESTINAL TRACT

It is estimated that 1-1.5 L of saliva is produced daily in humans, most of which is swallowed[15]. Thus, the human oral cavity is a constantly available source, transporting *C. concisus* from the oral cavity along with saliva to lower parts of the gastrointestinal tract. However, as mentioned previously, both the detection of *C. concisus* by PCR and the cultivation of *C. concisus* from fecal samples were much lower than that from saliva samples, indicating that *C. concisus* routinely transported from the oral cavity to the intestines does not commonly establish colonization there.

Nevertheless, intestinal colonization of oral *C. concisus* strains does occur in some individuals. For example, in a study comparing the housekeeping genes of *C. concisus* strains isolated from intestinal biopsies of patients with IBD and controls, Ismail *et al*[13] found that a *C. concisus* strain isolated from intestinal biopsies of a patient with UC had housekeeping genes identical to that of an oral *C. concisus* strain isolated from the same patient, providing evidence that the oral *C. concisus* strains are in fact able to colonize the human intestinal tract (Table 2).

In addition to an individual's own oral *C. concisus*, *C. concisus* detected in the intestinal tract may also come from a different source, most likely through materials contaminated with saliva from others. For example, in the above patient with UC, while the *C. concisus* strain isolated from intestinal biopsies had housekeeping genes identical to that of the patient's own oral *C. concisus* strain, the *C. concisus* strain isolated from the luminal fluid of this patient had a genetic relationship closely related to an oral *C. concisus* strain from a healthy control, rather than to the patient's own oral *C. concisus* (Table 2)[13].

The human intestinal environment is not optimal for *C. concisus* colonization in general, as suggested by the low isolation rate of *C. concisus* from fecal samples of healthy individuals (Table 1). Given this, *C. concisus* intestinal colonization is most likely a short-term event in most individuals. However, with the human oral cavity as a constantly available source of *C. concisus,* repeated intestinal colonization of *C. concisus* may occur.

Whether the characteristics of a given oral *C. concisus* strain or the intestinal environment of an individual, or both, plays the major role in determining whether or not intestinal colonization of oral *C. concisus* strains will occur is currently unknown. A previous study by Haag *et al*[16] showed that intestinal microbiota shifts towards elevated commensal *Escherichia coli* loads abrogated colonization resistance against *Campylobacter jejuni* in mice. Currently, it is not clear whether the dysbiosis associated with IBD plays a role in *C. concisus* intestinal colonization.

PREVALENCE OF *C. CONCISUS* IN THE INTESTINAL TRACT OF PATIENTS WITH IBD AND HEALTHY CONTROLS

A number of studies have examined the prevalence of *C. concisus* in the intestinal tract of patients with IBD using PCR methods. Most of these studies detected a significantly higher prevalence of *C. concisus* DNA in patients with IBD and controls[17-21]. The reported detection rates of *C. concisus* by PCR in enteric samples (biopsies and fecal samples) were 33%-69% in patients with IBD and 2%-38% in controls[17-21]. Analysis of the data in these studies revealed a number of interesting findings.

Firstly, different PCR strategies affect the detection of *C. concisus* in enteric samples. This was best seen in the study conducted by Man *et al*[18], who compared the prevalence of *C. concisus* in fecal samples collected from 54 children with CD, 33 healthy controls and 27 non-IBD controls using two different PCR methods. The first PCR method employed campylobacter genus specific PCR (primers C412F/C1288R) and sequencing the PCR products to determine campylobacter species. The second PCR method was a nested PCR, using campylobacter genus specific PCR (primers C412F/C1288R) followed by *C. concisus* specific PCR (primers Concisus F/Concisus R). These two PCR methods yielded very different results in detection of *C. concisus* in the same samples. The prevalence of *C. concisus* in children with CD, healthy controls and non-IBD controls detected by *C. concisus* genus specific PCR

Table 3 Detection rates of *Campylobacter concisus* in fecal samples by two polymerase chain reaction methods

	Campylobacter genus PCR	Nested PCR
CD (n = 54)	19%	65%
Healthy controls (n = 33)	12%	33%
Non-IBD controls (n = 27)	0	37%

An example showing that different polymerase chain reaction (PCR) methods affect the detection of *Campylobacter concisus* (*C. concisus*) in intestinal samples. Data included in this table were from reference 18. Primers C412F and C1288R were used in campylobacter genus PCR. In nested PCR, PCR products amplified by primers C412F and C1288R were amplified again using *C. concisus* specific primers Concisus F and Concisus R. The nest PCR detected a significantly higher intestinal prevalence of *C. concisus* in patients with Crohn's disease (CD) compared to both healthy controls and non-inflammatory bowel disease (IBD) controls. The genus PCR detected a significantly higher intestinal prevalence of *C. concisus* in patients with CD compared to non-IBD controls, but not healthy controls.

was 19% (10/54), 12% (4/33) and zero (0/27) respectively. The nested PCR greatly increased the detection of *C. concisus* in the same cohort of samples, with the prevalence of *C. concisus* being 65% (35/54) in children with CD, 33% (11/33) in healthy controls and 37% (10/27) in non-IBD controls. The nested PCR, but not the genus specific PCR, detected a significantly higher prevalence of *C. concisus* in children with CD as compared to healthy controls[18] (Table 3). Indeed, in studies revealing a significant difference in intestinal prevalence of *C. concisus* between patients with IBD and controls, nested PCR was used to examine all of the samples or part of the samples[17-20].

Secondly, collection of multiple intestinal biopsies increases the detection of intestinal prevalence of *C. concisus*. A study by Mahendran *et al*[20] showed that in comparison to the collection of one biopsy, the collection of four biopsies from each individual greatly increased the detection of *C. concisus* (Figure 2).

Thirdly, despite the increased prevalence of *C. concisus* detected by PCR in the intestinal tract of patients with IBD, the isolation rates of *C. concisus* from intestinal biopsies of patients with IBD were low (3%-7.7%)[17,20,21]. This suggests that *C. concisus* detected in most of the enteric samples were at a low number or in a nonculturable state.

ADVERSE EFFECTS OF *C. CONCISUS* ON INTESTINAL EPITHELIAL CELLS

A number of adverse effects of *C. concisus* on intestinal epithelial cells have been described. Using *in vitro* cell culture models (Caco2 cells or HT-29 cells), epithelial adhesion and invasion, damage of the barrier function and up-regulation of Toll-like receptors-4 expression by *C. concisus* have been reported. Different strains showed varying degrees of ability to induce such adverse effects[13,22-25]. The underlying molecular mechanisms responsible for these effects have not yet been investigated.

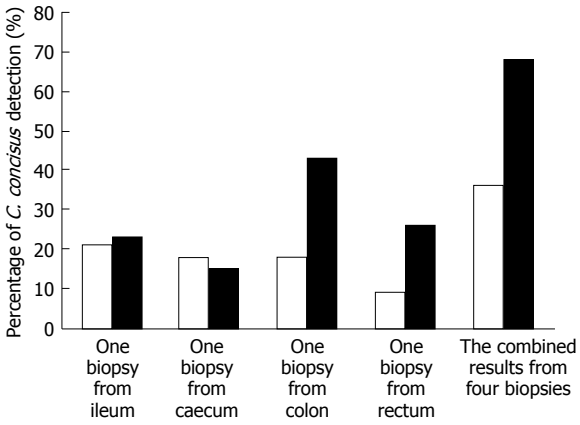

Figure 2 An example showing that collection of multiple intestinal biopsies increases the detection of intestinal prevalence of *Campylobacter concisus*. Data included in Figure 2 were from reference 20. In both patients with inflammatory bowel disease (IBD) (right column) and healthy controls (left column), the detection of intestinal prevalence of *Campylobacter concisus* (*C. concisus*) was greatly increased using four biopsies as compared to using one biopsy. In healthy controls, *C. concisus* detection rates in a single biopsy collected from ileum, caecum, colon and rectum were 21%, 18%, 18% and 9% respectively. If results from the four biopsies were used in determining the intestinal prevalence of *C. concisus*, the intestinal prevalence of *C. concisus* in healthy controls was 36%. Similarly, in patients with IBD, *C. concisus* detection rates in a single biopsy collected from ileum, caecum, colon and rectum were 23%, 15%, 43% and 26% respectively, and the intestinal prevalence of *C. concisus* in patients with IBD was 68% when four biopsies were taken into consideration.

C. CONCISUS ZOT GENE AND IBD

Mahendran *et al*[14] examined the prevalence of *zot* gene in 56 oral *C. concisus* strains isolated from saliva of 19 patients with IBD and 20 healthy controls. This study showed that 30% (17/56) of the oral *C. concisus* strains carried the *zot* gene. The *zot*-positive *C. concisus* strain was present in 55% (6/11) of patients with active IBD and 40% (8/20) of healthy controls. Some IBD patients with active disease 18% (2/11) were colonized with multiple *zot*-positive *C. concisus* strains in the oral cavity. Interestingly, polymorphic forms of the *C. concisus zot* gene resulting in the substitution of valine at amino acid position 270 were found to be associated with active IBD.

The *zot* gene was first discovered in *Vibrio cholerae* where it is carried by a filamentous prophage[26,27]. The *zot* gene in *V. cholerae* is required for phage production; a *V. cholerae* strain with *zot* gene mutation did not produce phage particles into the culture supernatant[28]. The *V. cholerae* Zot toxin was shown to increase intestinal permeability and to be associated with mild to moderate diarrhea[26,29,30].

Previous studies have found a human intestinal Zot analogue, namely zonulin, which is a physiological regulator that increases the intestinal permeability[31,32].

C. CONCISUS ZOT GENE IS A COMPONENT OF A CHROMOSOMALLY INTEGRATED PUTATIVE PROPHAGE

Stothard *et al*[33] established a visual database of bacterial

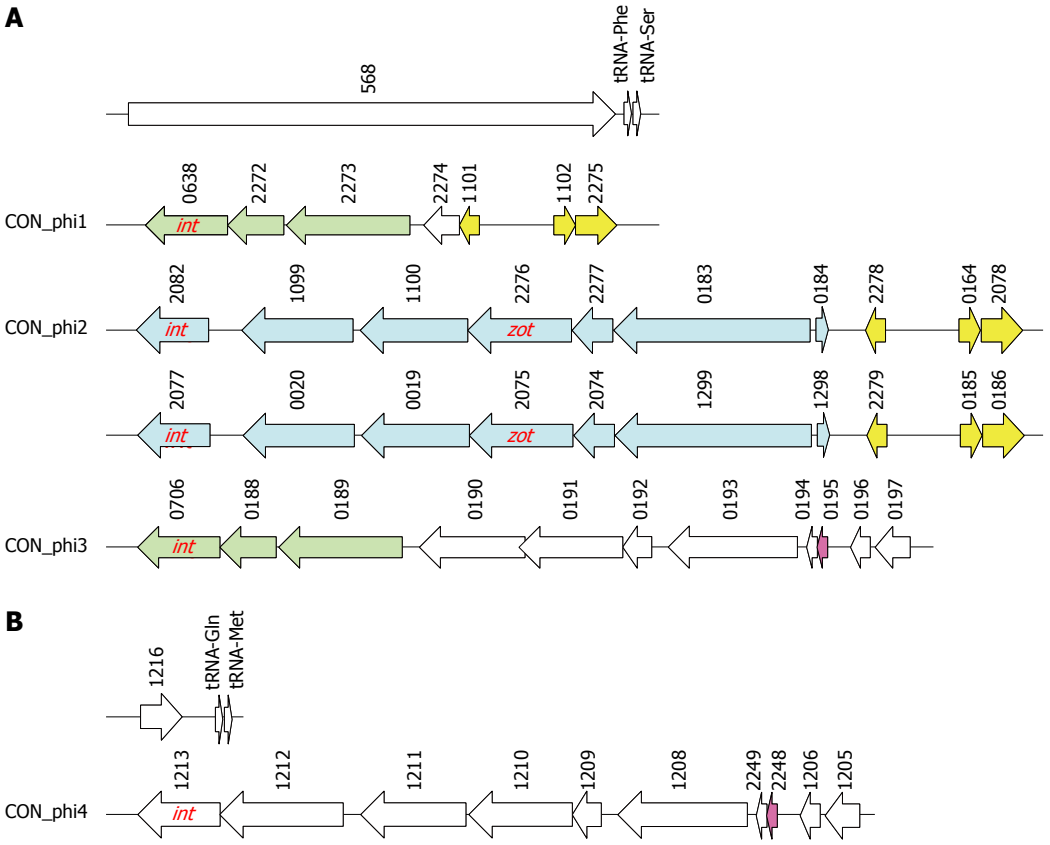

Figure 3 Genetic structures of putative prophage identified in *Campylobacter concisus* strain 13826. A: Multiple prophages at nucleotide position between 1576686 and 1614075; B: One putative prophage at nucleotide position between 939158 and 946901. Identical genes were indicated with the same color. *int*: A gene encodes phage integrase; *zot*: Zonula occludens toxin gene. The numbers are the locus tags.

chromosome maps in which *C. concisus* strain 13826 (Accession No. CP000792. 1) was included. Stothard *et al*[33] identified a region from nucleotide position 1576683 to 1615449 (38767 bp) in the genome of *C. concisus* strain 13826 as an "incomplete prophage". In this region, 39 genes with open reading frames were identified, with four of these genes encoding integrases and 10 genes encoding phage-like proteins[33].

The genetic structures of this "incomplete prophage" region are shown in Figure 3A and the proteins encoded by genes in this region are shown in Table 4. We compared the genes within this region using publicly available softwares[34,35]. A number of genes that have identical nucleotide sequences, including CCC13826_1099, CCC13826_0020, CCC13826_1102, CCC13826_0164, CCC13826_0185, CCC13826_2082 and CCC13826_2077 were annotated with identical protein names.

Interestingly, the region that was considered as an "incomplete prophage" by Stothard *et al*[33] turned out to be four putative prophages, each beginning with a phage integrase (Figure 3 and Table 4). The first prophage had a genome size of 5.2 kb, which contained seven protein-encoding genes. The second prophage and third prophage were identical, each having a genome size of 9.6 kb consisting of 10 protein-encoding genes. The fourth prophage contained 11 protein-encoding genes with a genome size of 8.6 kb. We named the first prophage CON_phi1, the second prophage and third prophage CON_phi2 and the fourth prophage CON_phi3. The *zot* gene is a component of CON_phi2. Comparison of the proteins encoded by genes in CON_phi2 with that encoded by genes in CTX phage, the phage that carries the *zot* gene in *V. cholera,* did not show high similarities except for the Zot protein (data not shown), suggesting the CON_phi2 is a previously uncharacterised prophage.

A study by Kaakoush *et al*[36] found that two hypothetical proteins encoded by CCC13826_0191 and CCC13826_1210 have 47% and 46% similarity respectively to *C. concisus* Zot. Here we found that CCC13826_0191 is a gene of CON_phi3 and CCC13826_1210 is a gene of an additional putative prophage, which was named CON_phi4. A number of genes in CON_phi3 and CON_phi4 had high similarities, however, CON_phi4 did not contain the gene that corresponding to CCC13826_0188 in CON_phi3 (Table 5).

IDENTIFICATION OF CONSERVED MOTIFS SHARED BY *C. CONCISUS* ZOT AND ZONULIN/ZOT RECEPTOR BINDING DOMAINS

Kaakoush *et al*[36] compared Zot sequences in *C. concisus* strain 13826 and *V. cholerae* strain 86015 and reported that

Table 4 Proteins encoded by putative prophages in *Campylobacter concisus* strain 13826

Nucleotide position	Locus tag	Encoded proteins	Size (aa)
1576686..1581986	CCC13826_0568	Hypothetical protein	1766
1582376..1583269	CCC13826_0638	Phage integrase	297
1583269..1583880	CCC13826_2272	Glutathionylspermidine synthase family	203
1583895..1585235	CCC13826_2273	Bacteriophage replication gene A protein	446
1585467..1585853	CCC13826_2274	Protein yitk	128
1585894..1586115	CCC13826_1101	Phosphonate uptake transporter	73
1586807..1587043	CCC13826_1102	Sensory box protein	78
1587044..1587496	CCC13826_2275	Hypothetical protein	150
1587598..1588506	CCC13826_2082	Phage integrase	302
1588755..1589966	CCC13826_1099	Putative phage replication protein A	403
1590015..1591184	CCC13826_1100	ATP binding protein (ABC) transporter	389
1591228..1592352	CCC13826_2276	Zonula occludens toxin (Zot) family protein	374
1592354..1592800	CCC13826_2277	Hypothetical protein	148
1592797..1594923	CCC13826_0183	Hypothetical protein	708
1594955..1595095	CCC13826_0184	Hypothetical protein	46
1595477..1595698	CCC13826_2278	Phosphonate uptake transporter	73
1596412..1596648	CCC13826_0164	Sensory box protein	78
1596649..1597101	CCC13826_2078	Hypothetical protein	150
1597203..1598111	CCC13826_2077	Phage integrase	302
1598360..1599571	CCC13826_0020	Putative phage replication protein A	403
1599620..1600789	CCC13826_0019	ABC transporter	389
1600833..1601957	CCC13826_2075	Zot family protein	374
1601959..1602405	CCC13826_2074	Hypothetical protein	148
1602402..1604528	CCC13826_1299	Hypothetical protein	708
1604560..1604700	CCC13826_1298	Hypothetical protein	46
1605082..1605303	CCC13826_2279	Phosphonate uptake transporter	73
1606017..1606253	CCC13826_0185	Sensory box protein	78
1606254..1606706	CCC13826_0186	Hypothetical protein	150
1606808..1607701	CCC13826_0706	Phage integrase	297
1607701..1608312	CCC13826_0188	Glutathionylspermidine synthase family	203
1608327..1609667	CCC13826_0189	Bacteriophage replication gene A protein	446
1609899..1611041	CCC13826_0190	Type Ⅱ and Ⅲ secretion system protein	380
1610992..1612116	CCC13826_0191	Hypothetical protein	374
1612118..1612438	CCC13826_0192	Hypothetical protein	106
1612537..1613946	CCC13826_0193	Hypothetical protein	469
1613956..1614075	CCC13826_0194	Alkyl hydroperoxide reductase	39
1614090..1614209	CCC13826_0195	Hypothetical protein	39
1614489..1614707	CCC13826_0196	Hypothetical protein	72
1614801..1615178	CCC13826_0197	Hypothetical protein	125

aa: Amino acids.

the biological active domain (FCIGRL) previously found in *V. cholerae* Zot was not found in *C. concisus* Zot. In this review, we compared the sequence of *C. concisus* Zot with human zonulin receptor binding domain and *V. cholerae* Zot receptor binding domain previously reported[32,37]. Interestingly, we found that *C. concisus* Zot shares conserved motifs with both the human zonulin receptor binding domain and the *V. cholerae* Zot receptor binding domain (Table 6). These data suggest that *C. concisus* Zot may increase intestinal permeability using a mechanism that is similar to the human zonulin and *V. cholerae* Zot, affecting the tight junctions through proteinase activated receptor 2 activation[37,38]. The motif "GRFLSYHG" is located at amino acid position 123-130 in *C. concisus* Zot, which was found in all oral *zot*-positive *C. concisus* strains that we previously isolated as well as in the *C. concisus* strain 13826[14]. The polymorphisms of *C. concisus zot* gene that Mahendran *et al*[14] previously detected were not in the receptor binding domain, suggesting that these polymorphisms may impact on the function of *C. concisus* Zot, if there is any, using a different mechanism rather than affecting the binding of *C. concicsus* Zot to the receptor.

INCREASED INTESTINAL PERMEABILITY IN PATIENTS WITH IBD

Increased intestinal permeability is a feature of both CD and UC[39-43]. While epithelial cell death and proinflammatory cytokines may damage the intestinal epithelial barrier during active disease, evidence shows that increased intestinal permeability may precede the initial onset or relapse of IBD. An early study from Hollander *et al*[39] reported that increased intestinal permeability was detected not only in patients with CD but also in their healthy relatives. A family history of IBD is a known risk factor for IBD[44]. Irvine *et al*[41] reported that an individual with a family history of CD had elevated intestinal permeability eight years prior to the onset of clinical symptoms and diagnosis of CD. Wyatt *et al*[43] measured the intestinal

Table 5 The similarity of proteins in CON_phi3 and CON_phi4

CON_phi3	CON_phi4	Similarity[1]
CCC13826_0706	CCC13826_1213	23.23%
CCC13826_0188		
CCC13826_0189	CCC13826_1212	93.72%
CCC13826_0190	CCC13826_1211	98.95%
CCC13826_0191	CCC13826_1210	92.25%
CCC13826_0192	CCC13826_1209	92.03%
CCC13826_0193	CCC13826_1208	58.90%
CCC13826_0194	CCC13826_2249	100%
CCC13826_0195	CCC13826_2248	97.44%
CCC13826_0196	CCC13826_1206	61.11%
CCC13826_0197	CCC13826_1205	95.24%

[1]Similarity is the percentage of identical amino acids.

Table 6 Motifs shared by *Campylobacter concisus* zonula occludens toxin and zonulin/zonula occludens toxin receptor binding domains

C. concisus Zot[1]	**GRF LSYHG**
Human adult intestine zonulin	**GGXL**
Human fetal intestine zonulin	**GGVLVQPG**
C. concisus Zot[2]	**GRF LSYHG**
V. cholerae Zot	FC**IGRLCVQDG**

[1]*Campylobacter concisus* zonula occludens toxin (Zot) and zonulin binding domain shares a motif (bold letters): Non-polar G, variable, non-polar, non-polar L, variable, polar, variable and non-polar G; [2]*C. concisus* Zot and *Vibrio cholerae* Zot shares a motif (bold letters): non-polar G, basic polar R, non-polar, non-polar, variable, polar, variable and non-polar G. Comparison of *C. concisus* Zot and zonulin/Zot receptor was performed in this review. The amino acid sequences of human intestinal zonulin and *V. cholerae* Zot were obtained from reference 32.

permeability in patients with quiescent CD and found that those with increased intestinal permeability were at a significantly higher risk of clinical relapse. These data suggest that increased intestinal permeability occurred prior to the onset and relapse of the disease may be a possible aetiological factor of IBD.

C. CONCISUS ZOT: A POTENTIAL TRIGGER OF IBD THROUGH CAUSING PRIMARY BARRIER DEFECT

The human zonulin and *V. cholerae* Zot toxin are known to increase intestinal permeability through affecting the tight junctions[31,32,37]. In this review, we found that *C. concisus* Zot has conserved motifs shared by the zonulin/Zot binding receptor domains. Given this, it is very likely that *C. concisus* Zot also affects the tight junctions.

Based on the information obtained from previous publications and the analysis that we have performed in this review, we propose a mechanism by which *C. concisus*, an oral bacterium, may trigger the onset or relapse of IBD: that some oral *C. concisus* strains acquire *zot* gene from a virus (prophage). With the human oral cavity as the reservoir of *C. concisus*, repeated intestinal colonization of *C. concisus* and release of *C. concisus* Zot due to prophage induction may occur, which is likely to result in a prolonged primary epithelial barrier defect and translocation of macromolecule such as luminal microbes and their products. In genetically susceptible individuals, this may trigger the development of IBD.

Damage to the intestinal epithelial tight junctions may also lead to the development of diarrhea. Indeed, in addition to its association with IBD, *C. concisus* has been frequently isolated from non-IBD-related diarrheal stool samples[9,45,46].

If some oral *C. concisus* strains are indeed involved in the development of human IBD, the question as to why the lesions of IBD occur more often in the intestinal tract rather than in the oral cavity arises. One explanation is that the virulence factors that are associated with IBD are more often expressed in the intestinal tract rather than in the oral cavity. For example, the expression of *C. concisus* Zot may require induction of prophage from the *C. concisus* genome. As prophage induction usually occurs when bacterial cells are under stressful conditions[47], the fact that *C. concisus* uses the human oral cavity as its primary colonization site suggests that the oral cavity is not a stressful site for *C. concisus*. However, as the *C. concisus* travels to the more hostile lower parts of the gastrointestinal tract, the stressful environment may trigger the induction of *C. concisus* prophage.

Another possible factor that may reduce the pathogenic effect of *C. concisus* Zot in the oral cavity is that the epithelium in the oral cavity is a stratified squamous epithelium, either keratinized or non-keratinized[48]. In contrast, the intestinal epithelium is a simple columnar epithelium[48]. The impact on permeability caused by Zot, even it is expressed in the oral cavity, in multiple layers of squamous epithelium may not be as evident as that in the single layered columnar epithelium.

C. CONCISUS ZOT: A POTENTIAL ENVIRONMENTAL FACTOR CONTRIBUTING TO THE INCREASED RISK OF IBD IN INDIVIDUALS WITH A FAMILY HISTORY OF IBD

A family history of IBD is a risk factor for developing IBD[44]. In addition to genetic factors, environmental factors have been shown to be involved in the increased incidence of IBD in members with a family history of this disease[49,50]. We suggest that *C. concisus* Zot is one such factor. This suggestion is based on the findings that the higher numbers of the relatives of patients with IBD have increased intestinal permeability and that some oral *C. concisus* strains carry the *zot* gene that encodes a toxin known to promote this[14,39,41]. This hypothesis remains to be further assessed by examining the correlation between colonization of *zot*-positive *C. concisus* strains and the increased intestinal permeability in family members of patients with IBD.

CONCLUSION

The evidence presented in this review shows that some *C. concisus* strains colonizing the human oral cavity acquired *zot* gene from a virus (prophage). We are currently examining the biologic activities of *C. concisus* Zot, the expression of Zot in *zot*-positive *C. concisus* strains isolated from patients with IBD and controls as well as the presence of *C. concisus* Zot in the oral cavity and intestinal tract of patients with IBD and controls, which will provide further information in understanding the role of *C. concisus* Zot in IBD and other human diseases.

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REVIEW

Novel hepatocellular carcinoma molecules with prognostic and therapeutic potentials

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Abstract

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is the sixth most common cancer worldwide and the third leading cause of cancer-related death. The difficulty to diagnose early cancer stages, the aggressive behaviors of HCC, and the poor effectiveness of therapeutic treatments, represent the reasons for the quite similar deaths per year and incidence number. Considering the fact that the diagnosis of HCC typically occurs in the advanced stages of the disease when the therapeutic options have only modest efficacy, the possibility to identify early diagnostic markers could be of significant benefit. So far, a large number of biomarkers have been associated to HCC progression and aggressiveness, but many of them turned out not to be of practical utility. This is the reason why active investigations are ongoing in this field. Given the huge amount of published works aimed at the identification of HCC biomarkers, in this review we mainly focused on the data published in the last year, with particular attention to the role of (1) molecular and biochemical cellular markers; (2) micro-interfering RNAs; (3) epigenetic variations; and (4) tumor stroma. It is worth mentioning that a significant number of the HCC markers described in the present review may be utilized also as targets for novel therapeutic approaches, indicating the tight relation between diagnosis and therapy. In conclusion, we believe that integrated researches among the different lines of investigation indicated above should represent the winning strategies to identify effective HCC markers and therapeutic targets.

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Key words: Hepatocellular carcinoma; Biochemical markers; Micro-interfering RNA; Epigenetic variations; Tumor stroma; Angiogenic factors

Core tip: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death. The modest efficacy of the therapeutic approaches also depends on the fact that the diagnosis often occurs in the late stages of the disease. Thus, the identification of early markers of onset and progression could be of significant benefit. Here we mainly focus on the latest data pointing the attention towards: (1) biochemical cellular markers; (2) micro-interfering RNA; (3) epigenetic variations; and (4) tumor stroma. The integration of these different lines of investigations should represent promising strategies to identify effective HCC markers.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer is the sixth most common cancer worldwide and the third leading cause of cancer-related death. Almost 80 percent of cases are etiologically related to chronic hepatitis B and C virus infection. Whereas the disease is most common in Asia and Africa, its incidence in the western countries is worrisome increasing. At present, the first line of curative treatment consists of the surgical resection or liver transplantation. The worldwide number of deaths per year in HCC is quite similar to the incidence number, underlying the very aggressive behavior of this disease as well as the poor effectiveness of first and second line of curative treatments. Additionally, as HCC is characterized by fast tumor cell growth, early hepatic metastasis, high grade malignancy and multidrug resistance, the 5-year survival rate is in the range of only 5%[1].

Effective treatment of HCC remains a big challenge also due to the lack of sensitive, early and specific diagnostic markers. Whereas the molecular mechanisms contributing to carcinogenesis and progression of HCC still need to be fully clarified, many biological molecules have been proposed as relevant players. Some of them are mutually exclusive of neoplastic transformation as in the case of eukaryotic elongation factor 1 A2, a protein normally expressed only by skeletal muscle, heart and nervous system[2-7]. Thus, a better understanding of the mechanisms underlying these signaling cascades may be of great diagnostic and therapeutic potentials.

A large number of biomarkers associated with HCC progression and aggressiveness has been proposed so far, but most of them turned out not to be of practical utility. This is the reason why the scientific community is actively investigating this field as demonstrated by the number of papers published in 2012 (740 items in PubMed using the key words: "hepatocellular carcinoma" and "marker"). Given the fact that the diagnosis of HCC typically occurs in the advanced stages of the disease when the therapeutic options have only modest efficacy, the possibility to identify early markers could be of significant benefit. In this regard, it should be pointed out that biomarkers can be searched in fresh/paraffin-fixed tissue specimens and in serum, targeting both proteins or nucleic acids[8].

Given the huge amount of published works aimed at the identification of HCC biomarkers, in this review we mainly focused on the data published in 2013 with particular attention to the role of (1) molecular and biochemical cellular markers; (2) micro-interfering RNAs (miRNAs); (3) epigenetic variations; and (4) tumor stroma.

MOLECULAR AND BIOCHEMICAL CELLULAR MARKERS

Table 1 resumes the most relevant findings with regard to the molecular and biochemical markers below described.

Glypican-3

Glypican-3 (*GPC3*) is a family member of heparan sulfate proteoglycan membrane proteins. It anchors to the cytoplasmic membrane *via* a glycosyl-phosphatidyl-inositol linkage. GPC3 can regulate in a positive or negative manner the activities of many growth factors depending on the cellular context[9]. In the adult, the expression of GPC3 can be detected in many types of cancers, including lung carcinoma and ovary adenocarcinoma. GPC3 was first proposed as a potential marker for hepatocellular carcinoma in 1997[10]. Nowadays it is considered as a relatively specific HCC biomarker being not detectable in hepatic para-carcinomatous and cirrhotic tissues[11]. It is interesting to note that more than 80% of HCCs overexpress GPC3[12]; additionally, GCP3 is also an interesting therapeutic target for HCC[9]. In this regard, a humanized monoclonal antibody against GPC3 has been recently developed[13] and it is under evaluation in a clinical trial phase Ⅱ in patients with advanced or metastatic HCC (http://www.cancerresearchuk.org/cancer-help/trials/trial-looking-gc33-advanced-liver-cancer). Moreover, it is worth mentioning that a vaccine GPC3-derived has been developed and tested in phase Ⅰ and Ⅱ clinical trials. The results of these studies are under evaluation[14].

GPC3 has been also proposed as a marker of HCC progression as it promotes cell growth by stimulating Wnt signal transduction pathway. Wnt is known to be involved in cancer acting as an oncogene able to induce the cytosolic accumulation and nuclear translocation of the transcription factor for the β-catenin; in turn, β-catenin activates the expression of genes promoting cell proliferation and survival[15]. The progression-associated meaning of GPC3 has been corroborated by a multivariate analysis on patients affected by HCC of different etiology where GPC3 resulted to be a predictor of poor prognosis[16]; however, in another study, the correlation was found only for HCC developed in HCV positive patients[17]. In a recent study of 172 patients undergoing liver resection, GPC3 was positively correlated with alpha-fetoprotein (AFP) levels and tumor node metastasis stage. Importantly, an high level of GPC3 in tissue was found to be an independent risk for a shorter post-operative disease-free survival, recurrence and for a decreased overall survival[18].

Dickkopf-1

Dickkopf-1 (DKK1), a secreted inhibitor of the Wnt/β-catenin pathway, is a negative regulator of bone formation and plays a role in cell proliferation and survival control. DKK1 is frequently overexpressed in many human tumors where it can promote or suppress tumor

Table 1 Molecular and biochemical markers of human hepatocellular carcinoma progression and poor prognosis

Molecule	Molecular category	Biological function	Expression levels in human HCC tissue or serum	Clinical pathological indications	Target for therapy	Ref.
GPC3	Member of heparan sulfate proteoglycan membrane proteins family	Regulates the signalling pathways of several growth factors, including Wnts	Over-expression; In tissue or serum (protein)	Metastasis, overall survival decrease, recurrence, prognostic value in early diagnosis	Yes	[12,14,16-18]
DKK1	Member of dickkopf family of secreted protein with two cysteine rich regions	Cell proliferation and survival control, differentiation, and cell motility; negative regulator of Wnt/β-catenin pathway	Over-expression in tissue (mRNA and protein) and in serum (protein)	Vascular invasion and metastasis, early diagnosis in AFP-negative HCC, distinguish between malignant and non-malignant liver diseases	Potential	[22]
S100A4	Member of S100 calcium-binding protein family	Target gene of Wnt/β-catenin pathway	Over-expression in tissue	Poorly differentiated HCC, vascular invasion and recurrence, overall survival decrease, up to now limited to HBV + HCC	Potential	[24]
S100 calcium-binding protein A14	Member of S100 calcium-binding protein family	Role not yet defined	Over-expression in tissue (mRNA and protein)	HCC vascular invasion and metastasis, disease-free and overall survival decrease	No data	[25]
Sex determining region Y (SRY) related high-mobility group box 6 (SOX6)	Member of the SRY box gene superfamily of transcription factors containing a DNA-binding high mobility group (HMG) domain	Involved in cell embryogenesis, differentiation and proliferation	Decreased expression in tissue (mRNA and protein)	Overall survival decrease, advance tumor stage	No data	[27]
SUOX	Mitochondria member of molybdenum oxotransferases superfamily	Involved in oxidative phosphorylation for ATP generation *via* electron transport chain	Decreased expression in tissue (protein)	Overall survival decrease in serum AFP positive, with increase of AKR1B10 and CD34 early diagnosis: high grade dysplastic nodules and HCC progression	No data	[28]
xCT	Component of cystine/glutamate transporter designated as system x_c^-	In mammalian cells involved in redox balance by mediating cystine entry in exchange for intracellular glutamate	Over-expression in tissue (mRNA)	Overall survival decrease related to the mRNA levels	Potential	[31]
GRK6	Member of the G protein-coupled receptor kinase of the Ser/Thr protein kinase family	Phosphorylates the activated forms of G protein-coupled receptors. It is involved in migration and invasion	Over-expression in tissue (protein)	Aggressiveness related to Ki-67, overall survival decrease	No data	[34]
GPR87	Member of the family of G protein-coupled cell surface receptor related to lysophosphatidic acid receptor family	Antiapoptotic role sustaining p53-mediated pro-survival of cells, involved in cell communication	Over-expression in tissue (protein)	Aggressiveness, intrahepatic metastasis related to CD133 up-regulation	Potential	[37]
MT-1 and MT-2	Members of the cysteine-rich, low molecular weight membrane proteins, localized on Golgi apparatus	Protect from oxidative damage and regulate metals homeostasis (storage, transport and detoxification). Involved in angiogenesis, apoptosis, cell cycle progression and differentiation	Decreased expression in tissue (protein)	Agressiveness, vascular invasion, recurrence free survival time reduced and overall survival decrease	No data	[39]
RAI3	Member of G-coupled cell surface receptor	Involved in the regulation of cell proliferation.	Over-expression in tissue (mRNA and protein)	Agressiveness, vascular invasion, increased tumor recurrence, shorter overall survival	Potential	[42]
SSX2IP	Spindle protein	Helps centrosome prepare for mitosis. Essential for centrosome maturation	Over-expression in tissue (mRNA)	Larger tumor size and metastasis, overall post-operative survival time reduced	Potential	[44]

Protein Phosphatase magnesium-Ppm1d	Member of PP2C family of Ser/Thr protein phosphatases	Inhibits p53 signalling, down-regulates p38 mitogen-activated protein kinase and thus is a putative oncogenetic protein	Over-expression in tissue (mRNA and protein), correlates with high AFP level	Larger tumor size, metastasis, shorter overall survival	No data	[46]
BCL9	Protein associated with B-cell acute lymphoblastic leukemia	Involved in signal transduction through Wnt pathway, promotes β-catenin's transcription activity	Over-expression in tissue (protein)	Aggressiveness, vascular invasion and metastasis. Shorter disease free status after hepatectomy	No data	[47]
IRF-1 and IRF-2	Belongs to transcription factors, member of the interferon regulatory factor family	IRF-1 and its functional antagonist IRF-2 are involved in the modulation of IFNs	IRF-1 decreased expression or IRF-2 over-expression in tissue (protein)	Aggressiveness, shorter overall survival and early recurrence	No data	[49]
CDK4	Member of the Ser/Thr kinase family	Controls the G1-S phase by phosphorylation of retinoblastoma protein	Over-expression in tissue (mRNA and protein)	Larger tumor size, HBV and shorter median survival	potential	[50]
LASP-1	Not yet elucidated	Involved in actin assembly	Positive cytosolic expression in tissue (protein)	Poorer survival	No data	[51]
PTP4A3	Member of PRL subgroup of protein tyrosine phosphatases	Involved in signaling pathways modulating cell proliferation, growth and motility	Over-expression in tissue (protein)	Overall survival decrease and shorter recurrence free survival	Potential	[52]
Steroyl-CoA-desaturase	Endoplasmic reticulum enzyme that catalyzed the biosynthesis of monounsaturated fatty acids	Catalyzed the D^9-*cis* desaturation of a range of fatty acyl-CoA substrates such as palmitoyl- and stearoyl-CoA (converted into palmitoleoyl-and oleyl-CoA, respectively)	Over-expression in tissue (mRNA)	Distinguishes between high and low risk HCC, positively related to AFP levels, tumor size and staging	Yes	[54]
PAK5	Member of the p21-activated kinase (Pak) family that serves as target for GTP-binding proteins involved in modulation of cell shape, movement, proliferation and survival	A mediator of filipodia formation	Over-expression in tissue (mRNA)	Early diagnosis and possibly indicator of HCC progression	Potential	[56]
hnRNP-L	Member of the hnRNPs that are involved in the processing of pre-mRNAs	Regulator of alternative splicing with both activator and repressor functions (retention and skipping of the exons)	Over-expressed (serum and tissue)	Progression in HBV-HCC patients, tumor size increase and overall survival decrease that correlates with high serum levels	Potential	[57]
CYLD	Protease, displaying endodeubiquitinase activity. Specifically cleaves 'Lys-63'-linked polyubiquitin chains.	Involved in the regulation of cell survival, proliferation, and differentiation. Plays an important role in pathways activating NF-κB and is a negative Wnt regulator. Involved in modulation of cell polarization, migration and angiogenesis	Downregulation at mRNA and protein levels (tissue)	Relates to resistance to chemotherapeutic agents	Yes	[58]
MAGE-D4	Component of the MAGE family protein	Expressed during development and involved in regulation of cell survival, cell cycle progression and apoptosis	Over-expression at mRNA and protein levels (tissue)	Relates to HCC poor differentiation and invasion. Marks early recurrence and decrease overall survival	No data	[59]

EphA3	Member of the receptor of tyrosine kinase family	Involved in development, particularly of the nervous system. Binds to ephrin-A ligands (especially EFNA5). Regulates cell-cell adhesion, cytoskeletal organization, cell differentiation and migration	Over-expression at protein level (tissue)	Decrease in overall survival	potential	[60]
Flot-1	Member of the Flotillin subfamily	Acts as a scaffolding protein within caveolar membranes and mediates cell signalings. Involves in the formation of caveolae and caveolae-like vesicles to regulate membrane trafficking. Participates to cytoskeleton organization, cell adhesion and invasion	Over-expressed at mRNA and protein levels (tissue)	High levels relates to increase tumor size, invasion and short disease-free time and overall survival	No data	[61]

GPC3: Glypican-3; DKK1: Dickkopf-1; S100A4: S100 calcium-binding protein A4; SUOX: Sulfite oxidase; xCT: Cystine/glutamate transporter; GRK6: G protein-coupled receptor kinase 6; GPR87: G protein-coupled receptor 87; MT-1: Metallothionein 1; MT-2: Metallothionein 2; RAI3: Retinoic acid-induced protein 3; SSX2IP: Synovial sarcoma X break point 2 interacting protein; Ppm1d: Protein phosphatase magnesium-dependent 1d; BCL9: B-cell CLL/lymphoma 9 protein; IRF-1: Interferon regulatory factor-1; CDK4: Cyclin-dependent kinase 4; LASP-1: LIM and SH3 protein 1; PTP4A3: Protein tyrosine phosphatase type IVA member 3; PAK5: P21-activated kinase 5; hnRNP-L: Heterogeneous nuclear ribonucleo protein L; CYLD: Cylindromatosis (turban tumor syndrome); MAGE-D4: Melanoma-associated antigen D4; EphA3: Ephrin receptor A3; Flot-1: Flotillin-1.

growth, depending on the tumor type[19]. In HCC, DKK1 expression was found to be significantly up-regulated. The identification of DKK1 in the serum allowed the detection of early stages of HCC development[20] and the identification of HCC in patients negative for the tumor marker AFP or to distinguish, in AFP positive patients, between HCC and chronic hepatitis B or liver cirrhosis[21]. In HCC tissue samples, the increased expression of DKK1 correlated with poor overall and disease-free survival, indicating its prognostic value[20]. Finally, as DKK1 up-regulation promotes invasion and metastasis in HCC, probably *via* a non-canonical Wnt pathway, it has been proposed as a useful therapeutic target[22].

S100A4

The up-regulation of S100 calcium binding protein A4 (S100A4), a member of the calcium-binding protein family, has been related to the metastatic cancer phenotype in a wide spectrum of cancers[23]. In HCC, a retrospective study showed that the expression levels of S100A4 correlated with tumor differentiation, invasion, recurrence, and overall survival. Notably, undifferentiated HCC tumors expressed higher levels of S100A4 compared to well-differentiated tumor forms. Additionally, increased levels of S100A4 were detected in HCC tumors with vascular invasion and recurrence[24]. It is worth mentioning that in this study the majority of the samples derived from patients with HBV-induced HCC; thus it has to be pointed out that the usefulness of S100A4 as a marker for HCC progression needs to be confirmed in other HCC types non promoted by HBV infection.

S100A14

A novel member of S100 protein is S100A14. In fresh HCC tissues with and without vascular invasion, the mRNA and protein levels of S100A4 were found to correlate with multiple tumor nodes and vascular invasion. Moreover, S100A14 detection correlated with both shortened disease-free survival and overall survival in HCC patients, thus proving its potential as prognostic factor. The role of S100A14 in sustaining HCC proliferation, migration and invasion was confirmed in HCC cell culture and *in vivo* (mice) analysis, thus supporting the role of S100A14 in sustaining HCC metastasis[25].

SOX6

SOX6 belongs to the high mobility group (HMG)-containing domain proteins whose biological role has been poorly investigated in humans so far. SOX proteins play part in embryogenesis regulating cell differentiation and proliferation. In particular SOX6 has a primary role in erythropoiesis and it seems to contribute to stem cell phenotype[26]. The decreased expression of SOX6 in HCC tissue samples, has been correlated with advanced tumor stage and less than 5-year-disease free overall survival in HCC patients. Notably, whereas in fresh and paraffin-embedded HCC human tissues SOX6 expression is down-regulated, in adjacent non-neoplastic tissues its level is un-affected. Therefore, reduced SOX6 expression is now considered a poor prognostic factor in HCC[27].

SUOX, AKR1B10 and CD34

Sulfite oxidase (SUOX), aldo-ketoreductase family 1 member B10 (AKR1B10) and CD34 markers, alone or in combination, have been very recently used for the differential diagnosis between high-grade dysplastic nodules and well-differentiated HCC. During hepatocarcino-

genic progression, SUOX levels were decreased, while AKR1B10 and CD34 showed elevated levels. Moreover, when combined with serum AFP, SUOX can independently predict post-surgical outcome, as well as the risk of tumor recurrence[28].

Cystine/glutamic acid transporter

Cystine/glutamic acid transporter (xCT) regulates the redox state in the cell by acting as an exchange system for cysteine/glutamine to produce glutathione (GSH), a major cellular antioxidant. GSH availability is limited by the amount of the sulfhydryl amino acid cysteine, which is readily oxidized to cystine and taken up by the Na^+-independent cystine/glutamate exchange transporter (system x_c^-). System x_c^- is composed of a light-chain subunit named xCT, which confers substrate specificity, and a glycosylated heavy-chain subunit (4F2hc or rBAT) common to the transporter family. xCT has been demonstrated to play a role in many cancers regulating the redox state of the tumor cells[29]. Recently, xCT has been proved to sustain the viability of the cancer stem cells[30]. In HCC fresh tissues it was found that xCT expression was higher than in normal liver tissues. Moreover, poor prognosis of HCC patients correlated with tumor size and the amount of xCT mRNA, indicating that xCT expression level can be used as a marker of HCC aggressiveness. Additionally, xCT has been proposed as a novel potential molecule for targeted therapy[31].

GRK6

G protein-coupled receptors belong to a superfamily of cell surface molecules known to regulate cell proliferation, migration and survival. G protein-coupled receptor kinase 6 (GRK6) is a member of the guanine nucleotide-binding protein (G protein); it is bound to the receptor kinase subfamily belonging to the Ser/Thr protein kinase family[32]. GRK6 regulates the activity of chemokine receptor and it is implicated in the modulation of cell adhesion[33]. A study performed using fresh and paraffin-embedded human tissue samples from HCC patients, demonstrated that GRK6 expression is higher in cancerous cells compared to the peri-tumor cells. Additionally, GRK6 expression level was positively related to the aggressiveness of the tumor and with the cell proliferation marker Ki-67. Finally, GRK6 expression level resulted to be negatively correlated with the survival rate. Thus GRK6 is considered a potential useful marker to predict prognosis of HCC patients[34].

GPR87

G protein-coupled receptor 87 (GPR87) can, among its functions, mediate p53 pro-survival effect[35]; additionally, it is frequently overexpressed in cancer[36]. GPR87 was found to be up-regulated in $CD133^+$ HCC cancer stem cells (CSCs) and to promote their survival *in vitro*. Notably, GPR87 favors CSCs migration and invasion as shown by the fact that GPR87 knock down significantly reduced these properties in CSCs. CSCs are proposed as key players in the development of different tumors, including HCC. For example, the knocking down of GPR87 expression resulted in the decrease of CSCs cell migration and invasion in mice[37]. Together, these data suggest a strong connection between GPR87 expression and CSCs, thus indicating GPR87 as a potential marker of HCC tumor development.

Metallothioneins

A group of intracellular metal binding proteins, called metallothioneins (MTs)-1 and -2 play distinct roles in metal homeostasis and in different cellular events including carcinogenesis[38]. The presence of MT-1 and -2 in the nuclei of rapidly growing cells is mainly correlated with their functions to provide zinc to the enzymes involved in the synthesis of nucleic acids or to chelate zinc from transcriptional factors, respectively. The expression of these two proteins is up-regulated in a variety of cancers, contributing to aggressiveness, poor prognosis, as well as to radiation and drug-resistance. In contrast to many other cancers, in HCC tissues, MT-1 and MT-2 expression, evaluated by microarray, has been found to be reduced, a fact correlated with a poor prognosis[39]. Additionally, reduced expression levels have been correlated to higher HCC grade and tumor vascular invasion[40]. Together these data indicate that the down-regulation of MT-1 and MT-2 may be undertaken as a poor prognostic factor for HCC patients.

Retinoic acid-induced protein 3

Retinoic acid-induced protein 3 (RAI3) is a member of G-protein-coupled receptors (GPCRs). It acts as a pivotal tumor suppressor or oncogene, depending on the human cancer type[41] where its expression can be either down or up-regulated. In HCC, increased expression of RAI3 has been associated with malignant progression and poor outcome after hepatic resection. Moreover, it has been considered an attractive candidate for targeted therapies[42].

Synovial sarcoma X breakpoint 2

Synovial sarcoma X breakpoint 2 interacting protein (SSX2IP), initially identified as an acute myeloid leukemia associated antigen, has been recently proved to play an important role in centromere maturation and thus in the correct spindle formation[43]. In HCC cell cultures and in mice, overexpression of SSX2IP promoted migration and invasion as well as drug-resistance. Additionally, the expression levels of SSX2IP in tumor tissues correlated with tumor size, tumor thrombosis and reduced survival of HCC patients. Thus SSX2IP may represent an interesting marker for HCC progression and a new target for therapeutic treatments[44].

Protein phosphatase magnesium-dependent 1 delta

Protein phosphatase magnesium-dependent 1 delta isoform (Ppm1d) also known as Wip 1 (wild type p53 induced protein phosphatase 1) belongs to Ser/Thr protein phosphatase 2C (PP2C) family. Ppm1d is involved in G_2/

M transition and acts as a regulator of cell proliferation. In particular it inhibits p53 and p38 functions thus being recognized as a putative oncogene[45]. Ppm1d is overexpressed in fresh HCC tissues compared to non-cancerous liver tissues. Notably, high levels of PPMD1 mRNA correlated with tumor size and stage and with poor patient's prognosis. Thus PPMD1 can be considered an interesting potential marker of HCC progression[46].

BCL9

BCL9, a gene derived from the translocation t(1;14)(q21;q32), is overexpressed in B-cell acute lymphoblastic leukemia and plays a role in Wnt/β-catenin signaling pathway thus contributing to cancer development and progression. Immuno-histochemical staining of primary HCC tissues indicated that BCL9 protein overexpression is related to younger age, higher Edmondson grade, tumor microvascular invasion and intrahepatic metastasis. Moreover, increased expression levels of BCL9 correlated with a lower five-year disease free survival rate, thus making of this protein a novel potential marker of HCC prognosis[47].

Interferon regulatory factor-1 and 2

Interferon regulatory factor-1 and 2 (IRF-1, IRF-2) are transcription factors that mediate interferon (IFN) functions. As these two transcription factors compete for the same DNA sequence, they are each other functional antagonist. Many evidences confer to IRF-1 and 2 a role in tumor onset and progression: in particular, the first is considered a tumor suppressor while the second an oncogene[48]. In HCC tissues both IRF-1 and 2 proteins localize in the nucleus. Whereas low levels of nuclear IRF-1 correlate with a poor outcome, low levels of IRF-2 correlate with a favorable outcome. Moreover, analyzing HCC cancer cell lines with low, moderate or high metastatic potential, correlations between the ratio IRF-2/IRF-1 and cell phenotype were observed. Thus IRF-1 and IRF-2 expression and their ratio may be considered relevant with regard to HCC prognosis[49].

CDK4

CDK4 is a member of the cyclin - dependent Ser/Thr protein kinase family that plays an important role in G_1-S cell cycle phase progression by phosphorylating the retinoblastoma protein. CDK4 overexpression has been observed in many cancers. In HCC tissues, the analysis of mRNA and protein expression showed that CDK4 is frequently (68%) overexpressed. Notably, CDK4 expression directly correlates with tumor stage, size and poor survival rate[50].

LASP-1

LIM and SH3 protein 1 (LASP-1) is a protein of not yet well-characterized functions. It is known to participate in cell motility and its up-regulation has been associated with invasive cancers. The immune-reactivity of cytosolic LASP-1 in HCC tissues correlates with overall patients' poor survival. *In vitro* LASP-1 was found to promote migration of HCC cell lines and its silencing strongly reduced migration. Thus, these findings suggest that the cytosolic elevation of LASP-1 levels may represent a marker of poor outcome and a potential target for therapeutic approaches[51].

PTP4A3

Protein tyrosine phosphatase type IVA member 3 (PTP4A3) is also known as phosphatase of regenerating liver-3 (PRL-3). It belongs to the PRL family of enzymes that regulates the signaling cascade for the maintenance of cellular viability and growth. By microarray gene expression assay, PTP4A3/PRL-3 was found to be overexpressed in poorly differentiated HCC tissues; additionally, its overexpression was related to a reduced overall survival. The close association between PTP4A3/PRL-3 expression and HCC progression, invasion and metastasis suggests that PTP4A3/PRL-3 could be a prognostic marker of poor outcome[52].

Fatty acid

The lipid metabolites derived from the activity of the stearoyl-CoA-desaturase (SCD) enzyme, a modulator of fatty acid flux, were independently associated with HCC outcome. The potential of SCD as a biomarker of poor outcome was demonstrated in a panel of HCC cell lines with increasing aggressive features. Additionally, high levels of SCD were detected in HCC specimens and were correlated with a short disease-free survival. Notably SCD expression was found to predict the prognosis both in HCC associated and HCC non-associated hepatitis B infection[53]. How SCD contributes to HCC aggressiveness remains to be defined. It has been suggested that the increased levels of the mono-unsaturated palmitoleate (MUPA), derived from the SCD enzymatic conversion of saturated palmitic acid (SPA), negatively affect Kupffer cell activation, cytokine expression and increase liver fibrosis. Finally an increased MUPA level promotes and sustains metabolic syndrome and inflammation, factors favoring tumor growth and progression[54,55].

PAK5

P21-activated kinase 5 (PAK5) represents the latest family member of P21-activated kinases (PAKs) with Ser/Thr kinase activity. Expression of *PAK5* gene in 25 out 30 HCC tissues has been demonstrated to be highly elevated with respect to the surrounding paraneoplastic tissue, making this gene an interesting diagnostic/prognostic marker for HCC[56].

hnRNPL

Humoral immunity response to tumor antigens (autoantibodies), known as tumor-associated antigens (TAAs), can occur in cancers and can be used to detect tumor presence. Heterogeneous ribonuclear protein L (hnRNP L) is a component of the hnRNP complexes involved in the processing of the pre-mRNA. By immune-proteomic

screening of HBV-HCC sera, hnRNP L has been recognized as a TAA in HCC. TAAs against the N-terminal glycine-rich region of hnRNP L (polypeptide sequence 67-88) were detectable in 60% of the serum samples from patients with HBV-related HCC but neither in normal controls nor in HBV-related liver cirrhosis serum samples. Interestingly, the titer of autoantibodies against hnRNP L 67-88 epitope, positively correlated with the increase in tumor size and with a reduced patients' survival. Moreover, in HCC cell cultures hnRNP L knocking down demonstrated that this protein sustains the growth, migration and invasion of the cancer cells. Thus the N-terminal epitope of hnRNP L is a potential biomarker for HCC progression at least in HBV-associated HCC and a potential candidate for HCC therapeutics[57].

Cylindromatosis gene

The tumor suppressor cylindromatosis gene (CYLD) codifies an ubiquitin carboxyl terminal hydrolase, a protease able to cleave Lys-63-linked polyubiquitin chains in proteins. CYLD plays important roles in the regulation of cell survival/proliferation/differentiation, as a pro-apoptotic factor and as a regulator of the pathways connected to NF-κB activation. In immuno-histochemical and tissue microarray analysis of HCC tissues, CYLD mRNA and protein levels were found to be considerably down-regulated in tumor sections with respect to surrounding non-malignant tissue. Interestingly, in cell cultures, the down-regulation of CYLD by siRNA was found to relate to an increase of HCC cells resistance to chemotherapy and to TNF-α-induced apoptosis. Moreover, in HCC cells, CYLD was found to be a negative modulator of NF-κB activity; importantly, CYLD expression can be restored by inhibition of EGFR-Raf-MEK-ERK signaling cascade as demonstrated by the use of EGFR tyrosine kinase inhibitor AG1478. Thus, CYLD could be a promising marker for HCC aggressiveness and progression, as well as an interesting target for HCC therapy[58].

Melanoma-associated antigen family protein D-4

Melanoma-associated antigen family protein D-4 (MAGE D-4), originally termed MAGE-E1, is a MAGE family gene expressed at high levels in malignant tumors. Overexpression of MAGE D-4 mRNA was found in 34 out 94 HCC tissue samples; additionally, the expression levels of MAGE D-4 as well as of the MAGE D4b protein were found to be directly related to HCC aggressiveness. Moreover, MAGE D-4 expression in combination with the AFP level, correlates with a poor tumor differentiation and vascular invasion, thus underlying the potential of MAGE-D4 as marker for early tumor recurrence and poor survival outcome[59].

EphA3

Ephrin receptors, the largest family of tyrosine kinases interacting with ephrins, are involved in a variety of cell functions including cell motility and adhesion. The ephrin receptor EphA3 is mainly localized in the cytoplasm and plasma membrane of cells. The down-regulation of EphA3 expression in HCC cell lines determines a decrease in the cell invasion capacity, suggesting its possible role as a therapeutic target. Finally, the overexpression of EphA3 in HCC tissues correlates with a decrease in the overall survival of patients[60].

Flotillin-1

Flotillin-1 (Flot-1) is a member of the membrane proteins belonging to the ubiquitary expressed lipid raft family. These proteins take part in the regulation of many cellular processes as membrane receptor signaling factors and regulate membrane trafficking, cytoskeleton organization, cell adhesion and migration. Flot-1 acts as a scaffolding protein within caveolae and is involved in the formation of caveolae; its de-regulation has been documented in many human cancers. Flot-1 was found overexpressed both at mRNA and protein levels in HCC cell lines. In HCC archive tissue samples, high level of Flot-1 protein correlates with an increase of tumor size, invasion, recurrence and shorter patients' survival time. Flot-1 levels thus could mark the aggressive HCC status and the poor prognosis of HCC patients[61].

MICRORNAS

In the last seven years the role of miRNAs in HCC development has become evident. MicroRNAs are small (approx 18-25 nt) non-coding double-stranded RNAs with the capacity to regulate the expression of target genes mainly by impairing mRNA translation, but also by inducing mRNA degradation[62,63] and by affecting chromatin remodeling[64]. The miRNA pathway starts with the transcription of a long precursor defined primary miRNA (pri-miRNA), which is subsequently processed (pre-miRNA) in the nucleus by a cellular enzyme called Drosha. The pre-miRNA is then exported from the nucleus to the cytoplasm where it is processed by the DICER enzyme complex to generate an approximately 22 bp long RNA duplex. The mature miRNA is then loaded onto RNA-induced silencing complexes (RISC) whose best-known component is the Argonaute (Ago2) protein. Within the two miRNA strands, the "sense" is discarded while the other, "antisense", is selected and used by RISC to bind complementary target RNA determining the so called "interference process". It should be noted that miRNA action tends to be highly redundant as multiple miRNAs can regulate the same transcript and a single miRNA can regulate several transcripts.

Due to their capacities to control gene expression behaving like tumor suppressors or oncogenes, miRNAs are considered to play a crucial role in carcinogenesis. The key role of many miRNAs in the control of cell proliferation, apoptosis and invasion underlies the relevance of their de-regulation in promoting cancer[65]. The frequent de-regulation of miRNAs in various human cancers, has lifted these small molecules to the ranks of attracting

pharmaceutical targets[66]. miRNAs are also considered amenable anticancer targets because of the wide spectrum of oncogenic targets they can control. There are two strategies to develop miRNA-based therapies: (1) microRNA antagonists, typically represented by antisense DNA oligonucleotides, which inhibit microRNAs by binding to the antisense strand of the miRNA; and (2) miRNA mimics, represented by short double stranded RNA molecules mimicking the miRNA of interest, which are used to simulate miRNAs function[67]. A relevant number of miRNAs have been proved to be down-regulated in HCC (see[68] for a comprehensive review). Here we focused on some new discoveries (Table 2) of miRNAs involved in HCC progression.

miRNA-122 is the best studied miRNA in liver; not only it plays an important role in maintaining the normal liver phenotype, its deregulation has been related to an increased risk to develop HCC[69]. Another HCC related miRNA is miR-181b. Upon HCC cells exposure to transforming growth factor (TGF)-β1, the expression of miR-181b is up-regulated with the consequent promotion of cell growth, survival, migration and invasion[70]. TGF-β1 can also induce the expression of miR-23a, 27a, and 24, which are involved in HCC cell growth and survival too[71].

In HCC cell lines and in HCC human tissue samples the overexpression of miR-18b is related to tumor progression and metastatic potential. Additionally, miR-18b overexpression is associated with a poor HCC prognosis. The tumorigenic effect of this miRNA seems to be related to the down-modulation of its target gene trinucleotide repeat containing 6B (TNRC6B), whose reduction promotes the metastatic potential of HCC cells. TNRC6B is a RRM-protein that mediates miRNA-guided mRNA cleavage[72]. Recently TNRC6B has been indicated to contribute to breast cancer metastasis probably acting like a transcription factor[73].

miR-372 regulates cell cycle, pro-survival signals and proliferation in cancer cells. In retrospective HCC human tissue samples, it was observed that miR-372 is overexpressed compared to the peri-tumor tissue; this observation was confirmed in HCC cell line compared to normal human hepatocytes. From the clinical point of view, miR-372 overexpression correlates with tumor aggressiveness and overall shorter patients survival. Because of all these observations, miR-372 has been suggested as a potential therapeutic target for HCC[74].

miR-650, a novel miRNA overexpressed in gastric cancer, promotes cell proliferation by binding to the inhibitor of growth family 4 (ING4)[75,76]. miR-650 was also found to be overexpressed in HCC tissues; notably, the highest levels were found in younger patients and in poor differentiated tumors.

miR-100 functions as a tumor suppressor and is often de-regulated in many cancers. Down-regulation of miR-100 in HCC tissues was found to positively correlate with higher tumor grade, metastasis and recurrence; it also represents an independent factor of poor prognosis. Moreover, *in vitro* up regulation of miR-100 was proved to inhibit HCC cell growth and to promote cell apoptosis *via* down-regulation of the polo like kinase-1. Thus it could be also an interesting new target for HCC therapy[77].

Recently it has been proposed that miRNAs may not only promote HCC when over-expressed in the cellular environment, but also when released into the extracellular environment by the producing cells. Thus, HCC cells can also condition the tumor microenvironment[78] (for a comprehensive review see[79]).

A mechanism through which miRNA expression can be de-regulated in HCC cells includes the modification of the methylation pattern of the miRNA promoters. For example, it was demonstrated that HBx, the oncoprotein encoded by the hepatitis B virus (HBV), causes the hyper-methylation of the promoter of miR-132 thus down-modulating its expression; this in turn results in the activation of Akt-signaling pathway which promotes HCC development in HBV positive patients[80].

The effects of miRNAs on HCC biology are not limited to miRNA up/down regulation. It is indeed possible that in the presence of physiological miRNA levels, the specific miRNA cannot control the expression of its target. This condition takes place when nucleotide polymorphisms/deletions/insertions/mutations occur either in the miRNA or in the target sequence. Since the binding of a miRNA to its target mRNA depends on a certain degree of complementarity, the presence of nucleotide polymorphisms/deletions/insertions/mutations may alter the binding capacity thus preventing/minimizing miRNA function. For example, a "TTCA" insertion disrupts the binding site for miR-122 and miR-378m to interleukin (IL)-1alpha leading to the up-regulation of IL-1alpha expression and the promotion of HCC development[81].

Recent studies indicate that among the molecular mechanisms de-regulating miRNA functions in tumors, a relevant role is played by the miRNA biogenesis-related genes (miRBir genes). These genes codify for proteins involved in miRNA processing and miRNA-induced silencing. Among these, it should be mentioned the Ago2 protein. Ago2 plays a paramount role in RNA-silencing as it cleaves the miRNA targeted mRNA[82]. *In vitro* and *in vivo* studies showed that Ago2 overexpression promotes HCC cell proliferation and migration. Moreover, mice injected by HCC cells with different aggressive phenotypes and containing the recombinant adenovirus harboring Ago2, developed tumors and metastasis. In contrast, non-visible or smaller tumors were found in control mice injected with the same HCC cells but containing an Ago2 empty adenovirus. Notably, the knocking down of Ago2 significantly reduced and in some cases suppressed cell tumor migration and metastasis. To further point towards a role of Ago2 in HCC, there is the observation that Ago2 is frequently found to be overexpressed in HCC tissues[83]. It seems that Ago2 tumorigenic potential may be due to the concomitant overexpression of the focal adhesion kinase (FAK), a protein tightly involved in tumor metastasis[83].

Ago2 is not the only miRBir genes involved in HCC.

Table 2 Micro-interfering RNA, miRBir and epigenetic markers of human hepatocellular carcinoma progression and poor prognosis

Molecule	Molecular category	Biological function	Expression levels in human HCC tissue	Clinical pathological indications	Target for therapy	Ref.
miRNA-18b	Belongs to Oncomir-1 or miR17-92 cluster	Involved in embryogenesis and spermatogenesis	Over-expressed	Poor differentiated HCC, high levels positively correlate with lower survival time	Potential	[70]
miRNA-372	Oncomir	Not well defined: it can act as an oncomir or as an anti-oncomir depending on the malignancy	Over-expressed	High levels positively correlate with advance TNM stage, shorter recurrence-free survival and overall survival	Potential	[74]
miRNA-650	Oncomir	Targets protein involved in cell proliferation and survival	Over-expressed	Higher levels in less than 60-year-old patients; levels positively correlate with HCC differentiation status	Potential	[75,76]
miRNA-100	Tumor suppressor	Regulates cell differentiation and survival	Down-regulated	Levels negatively correlate with tumor grade, metastasis and recurrence	Potential	[77]
miRNA-132	Tumor suppressor	Involved in the repression and control of inflammation and in neuronal morphogenesis	In HBV-HCC down-modulated by HBx protein causing promoter methylation	Levels of miR-132 inversely correlate with those of HBx in HBV-HCC patients. Early diagnosis of HBV-HCC	Potential	[80]
Ago2	Belongs to eIF2C/AGO subfamily	One of the effectors of RNA interference either at initiation or elongation phases, endowed with PIWI domain (endonuclease activity)	Overexpressed (mRNA and protein levels) and gene amplification	Can signed metastasis	No data	[83]
Dicer	Belongs to the RNase Ⅲ family	Cleaves double-stranded RNA and pre-miRNA in the short (20-25 nt) active miRNA	Down-regulated	Shorter recurrence-free survival time	No data	[84]
TFPI2 gene	Member of the Kunitz-type serine protease inhibitors gene	The encoded protein inhibits plasmin-and trypsin-mediated activation of matrix metallo-proteinases.	Up-regulation of gene promoter methylation (HCC tissues and serum)	TFPI2 methylation levels positively correlate with high grade TNM, improved detection of HCC in patients with AFP levels less than 400 μg/L	No data	[87]
Ten-eleven translocation protein 1 gene	Member of the TET family of methylcytosine dioxygenase enzymes gene	The encoded protein convert 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) in various embryonic and adult tissues	Up-regulation of promoter methylation	Low levels of TET 1 and 5hmC relates to shorter overall survival	No data	[89]
MAT1A, *MAT2A* genes	Member of the SAM synthesizing isozymes genes	The encoded enzymes are essential to SAM biosynthesis. MAT1A is expressed in mature liver, whereas MAT2A is expressed by fetal liver, extrahepatic tissues or in HCC.	Up-regulation of MAT1A promoter methylation and down-regulation of MAT2A promoter methylation	Based on in cell culture and animal models studies, decreased MAT1A/MAT2A expression ratio has been proposed as s poor prognostic factor in HCC patients	No data	[90]

TFPI2: Tissue factor pathway inhibitor-2; Ago2: Argonaute-2; MAT1A: Methionine adenosyltransferase; HCC: Hepatocellular carcinoma; HBx: Hepatitis B virus x; SAM: *S*-adenosylmethionine.

In this regard, it has been observed that Dicer and Ago3/Ago4 down-regulation is related to HCC promoting risks. In particular, their down-regulation was observed in non-cancerous samples derived from patients with smoking or alcohol intake habits. Finally, a decreased expression of Dicer genes was of prognostic significance of shorter recurrence-free survival time[84].

EPIGENETIC VARIATIONS

Modification of accessibility of the transcription machinery to gene promoters is one of the mechanisms regulating gene expression in mammalian cells. Although several mechanisms concur to regulate this process, the status of DNA methylation/de-methylation is of major relevance. DNA methylation is mediated by a class of enzymes called DNA methyltransferases which covalently link a methyl group to the cytosine residue within 5'-CpG-3' palindromes. While 5'-CpG-3' palindromes cluster (CpG islands) in the promoters of about half of all human genes, they are rare in other regions of the human genome, indicating their role in the regulation of gene expression. Following methylation, particular proteins containing a methylcytosine binding domain are recruited and linked to the methylated DNA, thus repressing gene transcription by blocking the access of transcription factors to the gene promoter. Thus, the preservation of the physiological methylation pattern of gene promoters is critical for the homeostasis of the normal cells. In contrast, alteration of promoter methylation patterns is commonly found in tumor cells including HCC cells[85] (Table 2). For example, the tissue factor pathway inhibitor-2

(TFPI2) gene encodes a serine protease inhibitor that is secreted into the extracellular matrix by specialized cells (*i.e.*, endothelial, smooth muscle, fibroblasts, keratinocytes and urothelium). Once secreted, it inhibits plasmin and trypsin-mediated activation of the matrix metalloproteinases (MMPs, see section 5.3 for more MMPs biological details). Inhibition of MMPs activation in turn results in a reduced invasion capacity of the tumor cell. Thus, the down-regulation of TFPI2 expression by promoter methylation reduces the inhibition of MPP activation favoring cell migration and metastasis. Notably, aberrant *TFPI2* promoter methylation has been detected in circulating cells of patients with colorectal cancer but not after complete surgical resection of the tumor[86]. In HCC serum samples, the degree of *TFP12* promoter methylation in circulating cell-free tumor DNA was found to positively relate to tumor stage[87]. Thus, *TFPI2* promoter methylation is becoming to be considered a potential marker for HCC diagnosis and progression.

In normal mammalian cells, the methylation pattern can be reversible; this phenomenon, although not yet elucidated in detail, depends on three enzymes ten-eleven translocation (TET)1, TET2 and TET3, which belong to the TET family. TET1-3 is necessary to convert the 5-methyl-cytosine (5mC) into 5-hydroxy-methyl-Cytosine (5hmC) to favor promoter de-methylation. Thus, promoter methylation status is based on the balance between 5mC and 5hmC[85]. In cancer cells 5hmC is frequently decreased compared to 5mC, indicating a hyper-methylated status of gene promoter[88]. The reduction of 5hmC in HCC tissue was observed in a rat model of diethyl-nitrosamine-induced liver cancer. Even more interestingly, in 146 human HCC tissue specimens, 5hmC was found to be significantly decreased in 69% of the samples compared to non-tumor tissues. Notably, patients in the 69% group were found to be associated with a shorter overall survival[89]. The fact that TET1 but not TET2 or TET3 proteins, was significantly decreased in HCC fresh human tissue specimens compared to non-tumor tissues, indicates a major role of TET1 over the other two members in regulating 5hmC/5mC balancing.

Methionine adenosyltransferase 1 alpha (MAT1A) gene encodes for an enzyme able to transfer the adenosyl moiety of ATP to methionine to form *S*-adenosyl-methionin (SAM). The liver is the most relevant source of SAM that in turn is the main source of methyl groups for biological methylations. The *MAT1A* gene products are iso-enzymes organized either in homo-tetramer (MAT Ⅰ) or in homo-dimer (MAT Ⅲ); a third iso-enzyme, MAT Ⅱ, is encoded by another gene named MAT2A. Both *MAT1A* and *MAT2A* genes can be epigenetically modulated. Down-regulation of MAT1A expression with the consequent reduction of the SAM levels induces HCC cell proliferation and promotes neovascularization. Since MAT Ⅰ Ⅲ:MAT Ⅱ ratio can significantly predict patient's survival, it has been proposed that expression ratio of MAT1A:MAT2A can be used as a potential prognostic marker for HCC[90]. Finally, liver MAT2A expression is substituted in adult life by MAT1A and the induction of the non-liver specific *MAT2A* gene is believed to contribute to hepato-carcinogenesis by stimulating liver cell proliferation[91].

TUMOR STROMA

During recent years, a large body of literature has considered the cross-talk between tumor cells and their surrounding stroma as a fundamental step in the process of HCC progression, epithelial-to-mesenchymal transition (EMT), tumor invasion and metastasis[92-95]. Tumor microenvironment (TME) is a dynamic system orchestrated by inflammatory and cancer cells, as well as stromal tissue (immune cells, fibroblasts, myofibroblasts, cytokines, and vascular cells), and extracellular matrix[96]. TME can affect various aspects of tumor biology, including development, progression and therapy response. Stromal cells contribute to the hallmarks of cancer by preserving proliferative signaling, preventing growth suppressors, arresting apoptosis, inducing angiogenesis, stimulating invasion and minimizing immune destruction[97,98]. Cell components of the TME can be originated either from nearby or distant tissues; this last event seems to be due to the fact that in the case of rapid cancer development, resident cells may be unable or numerically insufficient to support the requirements of tumor growth. In line with these findings, improving the knowledge on the cross-talk between cancer cells and their neighboring environment seems an emerging challenge for identifying novel molecular targets with diagnostic and prognostic potentials for many tumors including HCC.

TME, which plays a crucial role in the initiation and progression of HCC, mainly consists of: (1) a cellular component which includes hepatic stellate cells, fibroblasts, a vast range of immune cells such as tumor-associated macrophages and endothelial cells embedded into the extracellular matrix; (2) secreted factors including cytokines and growth factors such as TGF-β_1; and (3) proteolytic enzymes such as MMPs.

Cellular components

Among the components of tumor stroma, cancer-associated fibroblasts (CAFs), an heterogeneous population of stromal cells, are the most abundant cell type. During cancer progression, stromal fibroblasts, the major source of CAFs, undergo a process called "fibroblast to myofibroblast trans-differentiation[99]. This event is triggered by growth factors such as TGF-β, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), mainly secreted by malignant cells[100]. Recent findings indicate that CAFs can arise from mesenchymal stem cells (MSCs), which are recruited to the tumor milieu from both adjacent and distant tissues (either bone marrow or adipose tissue[101,102]). CAFs exhibit completely different morphological and functional characteristics compared to their normal counterparts: they contribute to diverse mechanistic aspects of cancer progression including ex-

tracellular matrix remodeling, suppression of immune responses and the secretion of growth factors and cytokines. Notably CAFs are very resistant against apoptosis[103]. Plenty of studies indicate the significance of numerous signaling pathways involved in the modulation of cancer-promoting characteristics of CAFs[100,104,105]. In this respect, targeting signaling pathways which modulate the cross-talk between CAFs and other cellular components of tumor milieu can be considered as a promising therapeutic strategy for cancer treatment[106].

Under physiological conditions, macrophages play a leading role in the process of wound healing. They also take a relevant part in tumors which can be considered as wounds that never heal[107]. Tumor-associated macrophages (TAMs), another relevant class of the TME cellular component, secrete cytokines and growth factors necessary for tumor tropism. Moreover, they have a fundamental role in the degradation of extracellular matrix, a fact that leads to the release of additional growth factors from the matrix and to the promotion of tumor cell invasion. Factors released by TAM not only influence tumor cells, but also TAM themselves. In this regard, cytokines such as IL-4[108] and IL-10, as well as TGF-β, found in the tumor milieu, can promote polarization of TAMs towards M2-activated cells. This last cell type, defined as M2 in comparison to the classically activated M1-type macrophages, are activated by glucocorticoid, IL-10 and cytokines such as IL-4/IL-13 and participate in HCC progression[109,110]. TAMs showing characteristics of M2 cells can further secrete a cocktail of cytokines and chemokines, which influence tumor cells and the surrounding environment. For example, M2 macrophages can secrete vascular endothelial growth factor (VEGF) or endothelial growth factor (EGF) inducing tumor angiogenesis. Additionally, by favoring the degradation of the extracellular matrix, TAMs contribute to the generation of an inflammatory microenvironment, which promotes tumor progression. Together, the above reported observations allow the conclusion that the presence of TAMs represents a maker of poor prognosis in cancers, including HCC[111,112]. Additionally, TAMs targeting can open a novel avenue towards innovative therapeutic approaches in various cancers, including HCC[113].

At the tumor site, the role of macrophages and monocytes is not limited to the generation and maintenance of a tumor inflammatory micro-environment, it also extends to a pro-angiogenic contribution. HCC is a highly vascularized neoplastic disease with the neotumor vessels functioning abnormally. Angiogenesis-promoting cell types, belonging to myeloid lineage (such as macrophages and monocytes), play multifaceted roles in the process of pathological angiogenesis. Angiogenic factors, among which we can nominate angiopoietin-2 (ANG-2), are secreted from HCC cells to stimulate tumor vascularization. Tie-2, a tyrosine kinase receptor of angiopoietins capable of binding to all members of angiopoietin family, is involved in the transduction of angiogenic signals. Immunofluorescence staining has revealed that Tie2 expressing monocytes/macrophages (TEMs) are actively present in human HCC tissues when compared to non-HCC tissues. Notably, the amount of TEMs is definitively superior at the HCC tumor site than in the peripheral blood. Moreover, significantly higher micro-vessel density at the site of HCC tumor correlated with the presence of TEMs, proposing a unique role for this population of tumor-infiltrating bone marrow-derived cells in the process of angiogenesis. It should be noted that TEMs are not peculiar of HCC; they are also detectable in a wide range of cancers, where angiogenesis is a determinant factor in cancer progression[114,115]. Together, this information makes the presence of TEMs an indicator of active angiogenesis in human HCC. Thus TEMs may be undertaken as a promising diagnostic marker for HCC[116].

Secreted factors

A wide spectrum of cytokines including various growth factors and tumor proliferating molecules has been demonstrated to contribute to HCC development and progression (Table 3). Hyeon *et al*[117] have shown that fibroblast growth factor 19 (FGF19) expression is correlated with early recurrence and poor prognosis in HCC. It has been previously demonstrated that FGF family members play key roles in development, angiogenesis, and cancer[118]. In HCC, over-expression of FGF19 contributes to the occurrence and progression of the disease; this is mainly due to the de-regulation of Wnt/β-catenin signaling which promotes tumor invasion and metastasis[119]. Notably, inactivation of FGF19 or its receptor, FGFR4, has been shown to reduce the risk of tumor development[120-122].

TGFβ-1, the most potent pro-fibrotic cytokine, is in large part produced by cancer cells. TGFβ enhances HCC progression through a paracrine mechanism. Under normal conditions, TGFβ-1 inhibits cell proliferation, induces cell differentiation or triggers apoptosis. However, during cellular transformation, inhibition of cell growth by TGFβ-1 is hampered, resulting in an excessive cell proliferation. During tumor development, TGFβ-1 plays a pivotal role in processes such as fibrogenesis, angiogenesis, immunosuppression, and invasiveness of neoplasia. In HCC patients, elevated plasma concentration of TGFβ-1 is correlated with shorter survival[123]. It is believed that TGFβ-mediated EMT plays a crucial role in the aggressiveness of HCC. In this regard, the TGFβ concentration has been considered as a potent negative prognosticator in un-resectable HCC patients[124]. TGFβ can induce EMT of malignant hepatocytes by stimulating CAFs proliferation. Once activated, CAFs modulate growth, intra-vasation and metastatic spread of HCC cells. Interestingly, TGFβ receptor inhibition can significantly reduce the stromal component of the tumors and the metastatic dissemination of HCC cells *via* the reduction of CAF proliferation[125]. TGFβ activation of CAFs seems to be mediated by the down-regulation of E-cadherin and by the up-regulation of Snail/PDGF

Table 3 Some secreted factors from cellular components of tumor microenvironment and their contribution to hepatocellular carcinoma prognosis

Molecule	Molecular category	Biological function	Expression in human HCC	Clinico-pathological indications	Target for therapy	Ref.
Interleukin-4	Cytokine	Alternative activation of macrophages into M2 cells	Over-expressed (elevated level in serum)	Associated with extrahepatic metastasis	Not presented	[108]
TGF-β1	Multifunctional cytokine	Proto-oncogene/tumor suppressor	Over-expressed (elevated level in serum)	Decreased overall survival, increased risk of metastasis and recurrence, positively correlated with vascular invasion, higher PVT, higher overall invasiveness	Potential	[123]
FGF19	Growth factor	Activation of mitogen-activated protein kinase; activation of Wnt/β-catenin pathway	Over-expressed in tissue	Decreased disease-free and overall survival, increased risk of metastasis, positively correlated with vascular invasion	Potential	[117]
Fox C1	FOX transcription factor family member	Induction of EMT, promotion of epithelial cell migration *via* inhibition of E-Cadherin transcription through transactivation of SNAI1 expression	Over-expressed in tissue	Increased risk of metastasis and recurrence, increased tumor size and number, poorer tumor differentiation, poorer TNM stage, higher vascular invasion	Potential	[130,131]
Lcn2	Member of lipocalin protein family	Inhibition of epithelial-to-mesenchymal transition; suppression of JNK and PI3K/Akt signaling pathways, down regulation of Twist1	Over-expressed in tissue	Positively correlated with poor prognosis and high risk HCC, decreased overall survival, however, elevated levels of Lcn2 is correlated with the inhibition of EMT *in vitro* and *in vivo*	Not presented	[134,135]
uPA	Serine protease	Plasminogen activation, extracellular proteolytic activity	Over-expressed in tissue	Decreased disease-free and overall survival, increased HCC invasion, poorer pathological grade	Potential	[144,146]
uPAR	Urokinase receptor	ECM degradation, migration, invasion and metastasis	Over-expressed in tissue	Decreased disease-free and overall survival, higher risk of HCC invasion, portal cancer embolus and tumor metastasis, poorer tumor cell differentiation	Potential	[145,146]
PAI-1	Protease inhibitor	Regulating extracellular matrix degradation	Over-expressed in tissue	Decreased disease-free and overall survival, increased risk of metastasis and recurrence, increased regional invasion, poorer pathological grade	Not presented	[144,146]
VEGF	Growth factor	Regulation of vascularization and angiogenesis	Over-expressed in tissue, elevated level in serum	Poorer overall survival, increased risk of metastasis and recurrence, higher vascular invasion	Potential	[156]
HIF-1α	Subunit of HIF-1 transcription factor	Activating the transcription of a variety of genes involved in angiogenesis, survival pathways, immune evasion, invasion, and metastasis	Over-expressed in tissue	Decreased disease-free (and overall) survival, increased risk of metastasis, higher vascular invasion	Potential	[162,163]

PVT: Portal vein thrombosis; EMT: Epithelial-to-mesenchymal transition; TNM: Tumor node metastasis; FGF19: Fibroblast growth factor 19; Fox C1: Forkhead box C1; Lcn2: Lipocalin-2; uPA: Urokinase plasminogen activator; uPAR: Urokinase plasminogen activator receptor; PAI-1: Plasminogen activator inhibitor type-1; VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia-inducible factor-1α.

signaling pathways[126,127]. Notably, the reduced expression of E-cadherin has been associated with poor HCC tumor outcome and shorter disease-free survival[128].

Other transcriptional regulators play a role in EMT modulation. For example, forkhead box C1 (FoxC1), a member of the FOX transcription factor family[129,130], is one of these. Fox proteins are key regulators of epithelial-to-mesenchymal transition. Accordingly, the fundamental role of EMT in the incidence of HCC invasiveness and metastasis strongly indicates the involvement of these transcriptional factors in HCC metastasis. Xia *et al*[131] have demonstrated that the expression of FoxC1 in HCC patients is associated with shorter overall survival time and higher risk of recurrence, thus representing a negative prognostic factor for HCC patients. FoxC1 contributes to EMT induction through the inhibition of E-Cadherin expression *via* the transactivation of Snai1 expression; alternatively, FoxC1 induces the up-regulation of a protein named "neural precursor cell expressed, developmentally down-regulated 9 (NEDD9)", a member of Cas family of signal transduction factors. In turn, NEDD9 contributes to integrin-dependent cell migration and invasion through the activation of FAK- and Src-signaling cascades[132]. In HCC patients, NEDD9 over-

expression is significantly correlated with shorter overall survival and higher rate of metastases. FoxC1 can also favor EMT *via* the interaction with Notch and VEGF pathways, thus facilitating tumor neovascularization and vessel maturation processes[133]. Together these observations indicate FoxC1 as an interesting prognostic biomarker and potential target for novel therapeutic strategies for HCC.

In HCC, lipocalin-2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin (NGAL), negatively modulates EMT acting on the EGF or TGFβ/Lcn2/Twist1 pathway[134,135]. Up to now, conflicting data have been reported for the biological functions of elevated Lcn2, stressing its neoplasia-specific effects. However, in HCC an increasing body of literature indicates Lcn2 as a prognostic marker. Wang *et al*[134] have demonstrated that HCC specimens express significantly higher levels of Lcn2 compared to the healthy adjacent liver tissues. In this study, Lcn2 expression was significantly associated with a worse differentiation tumor grade even though it negatively correlated with EMT. In line with this last observation, adenoviral transduction of Lcn2 into sarcomatoid hepatocellular carcinoma (SH-J1) cells reversed EMT *in vitro*, inhibited cell proliferation and invasion and suppressed tumor growth and metastasis in a mouse model of HCC, further stressing the anti EMT function of Lcn2. In contrast, Lcn2 knock-down in HKK-2, a cholangio-carcinoma cell line, promoted EMT. In fact, a large body of evidence indicates the role of Lcn2 in preventing invasion and metastasis *in vitro*[136-138]. However, elevated expression level of Lcn2 has been significantly correlated with a poor prognosis and shorter survival time. Whereas more knowledge about Lcn2 in HCC needs to be gained, the above observations propose Lcn2 as a possible metastasis suppressor and a potential therapeutic target in HCC[139].

Together, the above reported data support the concept that targeting the cross-talk between HCC and its TME can represent a promising approach for the development of novel prognostic and therapeutic strategies against HCC.

Proteolytic enzymes

The local microenvironment of a tumor cell has a fundamental role in cancer development. Extracellular matrix (ECM), the main component of this milieu, is composed of a complex mixture of macromolecules, each with unique multifaceted properties. ECM is the fundamental scaffold of liver architecture and is in constant interaction with its environment, playing key roles in signal transduction and gene expression variations[140]. Notably, ECM abnormalities affect the behavior of stromal cells in favor of tumor-associated angiogenesis, thus establishing a tumorigenic microenvironment. In cancer, ECM is de-regulated, leading to cancer progression by facilitating cellular transformation and metastasis. In order a tumor to develop and spread, cancer cells produce certain proteases responsible for the degradation of ECM. The components of ECM (collagen, elastin, and gelatin) are degraded by various proteolytic enzymes, which include MMPs[141]. MMPs are a family of enzymes characterized by their proteolytic ability to degrade the ECM. There are six main families of MMPs: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24 and -25), and other MMPs, which are not categorized in any of the previous groups. The MMPs may act either as secreted enzymes or as trans-membrane pro-enzymes that require activation after secretion. Accelerated breakdown of ECM occurs in various pathological processes, including inflammation, chronic degenerative diseases and tumor invasion.

Overexpression of MMP family members alone or together, has been shown to be associated with the recurrence, invasion and metastasis of HCC[142]. For instance, plasma levels of MMP-9 can predict the probability of vascular invasion in HCC patients[143]. Notably, urokinase plasminogen activator (uPA) is able to degrade ECM and, by converting plasminogen to plasmin, to activate MMPs. Increased activity of uPA has been shown to act as a sensitive and reliable marker, affecting HCC invasion, disease free survival and recurrence of HCC[144]. Additionally, elevated levels of the uPA receptor (uPAR)[145] and uPA inhibitor also known as plasminogen activator inhibitor (PAI-1) factor[146], correlate with tumor aggressiveness and poorer outcome in HCC patients, especially when all these three markers are overexpressed[144]. This finding, in contrast with the inhibitory effect of PAI-1 on uPA and thus on MMPs, indicates the complexity of the relation between matrix degradation/remodeling and tumor progression.

Angiogenic factors

Angiogenesis is the process of formation of new blood vessels from pre-existing ones. Since metabolically active cancer cells need oxygen and nutrients to survive, they secrete large amounts of pro-angiogenic factors that promote tumor neovascularization[98,147]. In the past, angiogenesis has been considered an important manifestation for cancer development only after the formation of macroscopically detectable tumors. However, recent evidence indicates that this process also significantly contributes to the microscopic phase of neoplastic formation, as observed in pre-malignant and non-invasive lesions of in situ carcinomas[148,149].

Rapid growth of tumor mass leads to increased oxygen consumption. Under such a condition, tumor cells face hypoxia. Cancer cells overcome the hypoxia imposed to the tumor microenvironment by two different strategies: promoting angiogenesis, or developing metastatic behavior as a key tool to escape from hypoxic condition[150]. A remarkable feature of most HCCs is hypervascularization, emphasizing the fact that HCC generates various angiogenic elements with a high tendency to invade the vasculature. Angiogenic response in HCC is regulated by an intricate network of growth factors acting

on both tumor and endothelial cell populations. So far, a wide range of angiogenic factors and their receptors have been considered as therapeutic targets for HCC. In particular, VEGFs, the most studied and potent pro-angiogenic factors, together with their receptors (VEGFRs), have been the main focus of anti-angiogenic cancer therapies for the last decades. Indeed, among key signal transduction pathways, VEGF/VEGFR system plays a crucial role in the pathogenesis of HCC and a variety of molecular targeted agents have been developed to target the process of angiogenesis by inhibitory molecules controlling VEGF pathway. For example, Sorafenib, the first oral multikinase inhibitor agent, has been demonstrated to improve the survival of patients by affecting tumor angiogenesis and proliferation[151]. In particular, Sorafenib inhibits cancer growth by targeting tyrosine kinase receptors including VEGFR-2 and VEGFR-3. It also inhibits various kinases in the MAPK pathway. However, the benefits of VEGF-targeted therapeutics in the treatment of advanced neoplastic diseases, including HCC, is hampered by multiple escape mechanisms[152].

Not only VEGF has been considered a potential interesting target for novel therapeutic approaches, it is also considered to have a prognostic value. Elevated plasma levels of VEGF have been considered a negative prognostic biomarker in patients with advanced HCC[153-156]. Higher expression level of tissue VEGF, a prominent characteristic of HCC, corresponds to neovascularization at the site of tumor, associates with poor outcome and disease free survival, and correlates with higher tumor grade and vascular invasion[155]. Additionally, increased expression of the $VEGF_{165}$ isoform has been demonstrated to be an independent prognostic marker of recurrence development[157]. High levels of VEGF in the serum of HCC patients are significantly associated with the presence of intrahepatic metastasis and microscopic venous invasion, and thus can be considered also a biomarker of tumor invasiveness[158,159].

In addition to VEGF, low oxygen concentration at the site of tumor, also induces the expression of hypoxia inducible factor 1 (HIF-1), a transcription factor composed by two subunits: HIF-1α, and HIF-1β. The expression of HIF-1α subunit is up-regulated under hypoxic conditions[160]. In contrast, HIF-1β is constitutively expressed. HIF-1α over-expression is a typical characteristic of malignant cells. This transcription factor, as a master mediator of hypoxic adaptation, activates the transcription of a variety of genes including those involved in angiogenesis, survival pathways, immune evasion, invasion, and metastasis. It has been demonstrated that both mRNA and protein levels of HIF-1α, can be used as potential prognostic factors for survival and recurrence in HCC patients[161-163].

In HCC, hypoxia triggers invasion and metastasis also by inducing EMT, a phenotypic modification that confers to tumor cells the ability to migrate and to invade other organs. In HCC, one of the mechanisms by which EMT is induced occurs *via* HIF-1α. This factor can promote tumor growth and dissemination through the regulation of some EMT modulators, such as E-cadherin, a protein known to stimulate anti-growth signals. More in detail, it has been proposed that HIF-1α mediates repression of E-cadherin expression through the up-regulation of E-cadherin-specific repressor SNAI1. In animal models of HCC, tumor progression was associated with the up-regulation of HIF-1α and sequential production of VEGF. Thus, blockade of HIF-1 might be considered as a promising therapeutic approach in cancer treatment[164]. In a study by Zhang *et al*[165], the expression levels of HIF-1α, together with SNAI1 and other EMT modulators, were assessed in a cohort of HCC patients. The authors demonstrated a significant correlation between the dual over-expression of HIF-1α and SNAI1 and the reduced disease-free survival and poor prognosis. Notably, shRNA-mediated suppression of HIF-1α reverses the process of EMT. Even more interestingly, EMT phenotype can be reversed by re-oxygenation[165]. This phenomenon occurs also in HCC cells cultured in normoxic, hypoxic and re-oxygenated conditions.

A large body of evidence suggests that hypoxia induced by anti-angiogenic therapy can stimulate the escape of cancer cells from oxygen-deprived areas to well-nourished distant organs. It has been proposed that the more hypoxic tumor environment induced by inhibitors of VEGF and its receptors may trigger an invasive metastatic switch[152,166]. However, this hypothesis needs further investigation[167]. Finally, in some cases, drug induced hypoxia to the tumor site may be responsible for the development of drug resistance behavior. In this regard, it has been observed that the intra-tumor hypoxia induced by Sorafenib therapy can cause Sorafenib resistance in HCC patients; interestingly, the concomitant inhibition of HIF-1α can enhance the antitumor effects of this drug[168].

CONCLUSION

The diagnosis of HCC typically occurs in the advanced stages of the disease when the therapeutic options have poor efficacy. Thus, the identification of specific markers for HCC diagnosis and prognosis is thought to improve the effectiveness of the therapeutic approach to HCC. Based on this, much effort has been put in the selection of reliable HCC markers. In addition to the study of the biochemical markers, of the angiogenic mediators and of the epigenetic variations, which have obtained promising results, alternative lines of research have been proposed. In particular, miRNAs, due to their capacities to control gene expression, behaving like tumor suppressors or oncogenes, are arising as very attractive molecules. Also very interesting and novel is the consideration dedicated to the tumor microenvironment, a crucial regulator of HCC initiation and progression. Improving the knowledge about the cross-talk between cancer cells and the neighboring environment may be very useful to design novel molecular targets with diagnostic and prognostic potentials in HCC.

In conclusion, the evaluation of a broad spectrum of different HCC markers can reasonably identify a range of effective diagnostic, prognostic and therapeutic targets for HCC in the near future. Whereas it is difficult to predict the timing required to reach the goal, we believe that an integrated multidisciplinary research among the different lines of investigation highlighted in this review should represent the winning strategy.

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ORIGINAL ARTICLE

Factors correlating with acoustic radiation force impulse elastography in chronic hepatitis C

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Abstract

AIM: To investigate the factors other than fibrosis stage correlating with acoustic radiation force impulse (ARFI) elastograpy in chronic hepatitis C.

METHODS: ARFI elastograpy was performed in 108 consecutive patients with chronic hepatitis C who underwent a liver biopsy. The proportion of fibrosis area in the biopsy specimens was measured by computer-assisted morphometric image analysis.

RESULTS: ARFI correlated significantly with fibrosis stage (β = 0.1865, P < 0.0001) and hyaluronic acid levels (β = 0.0008, P = 0.0039) in all patients by multiple regression analysis. Fibrosis area correlated significantly with ARFI by Spearman's rank correlation test but not by multiple regression analysis. ARFI correlated significantly with body mass index (BMI) (β = -0.0334, P = 0.0001) in $F0$ or $F1$, with γ-glutamyltranspeptidase levels (β = 0.0048, P = 0.0012) in $F2$, and with fibrosis stage (β = 0.2921, P = 0.0044) and hyaluronic acid levels (β = 0.0012, P = 0.0025) in $F3$ or $F4$. The ARFI cutoff value was 1.28 m/s for $F \geqslant 2$, 1.44 m/s for $F \geqslant 3$, and 1.73 m/s for $F4$.

CONCLUSION: ARFI correlated with fibrosis stage and hyaluronic acid but not with inflammation. ARFI was affected by BMI, γ-glutamyltranspeptidase, and hyaluronic acid in each fibrosis stage.

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Key words: Acoustic radiation force impulse; Body mass index; Chronic hepatitis C; Computer-assisted morphometric image analysis; Fibrosis stage; Hyaluronic acid; Liver stiffness measurement; Transient elastography; Velocity of shear wave

Core tip: The assessment of liver fibrosis stage is important to estimate prognosis and to identify the patients requiring antiviral treatment in chronic hepatitis C. Liver biopsy is a gold standard for assessing fibrosis, but is invasive. Thus methods for noninvasively assessing fibrosis have been developed. Liver stiffness measurement (LSM) by Fibroscan and acoustic radiation force impulse correlate with fibrosis stage. However, LSM may be affected by factors other than fibrosis, such as edema, steatosis, and inflammation.

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INTRODUCTION

The assessment of liver fibrosis stage is important to estimate prognosis and to identify the patients requiring antiviral treatment in chronic hepatitis C.

Methods for noninvasively assessing liver fibrosis have been developed. Liver stiffness measurement (LSM) by transient elastography (TE) with Fibroscan[1-3] and velocity of shear wave (Vs) measured by acoustic radiation force impulse (ARFI)[4-6] correlate with liver fibrosis stage in various liver diseases. However, LSM is affected by factors other than liver fibrosis, such as edema, steatosis, inflammation and necrosis. In particular, inflammation affects LSM; acute or chronic inflammation can result in a high LSM, indicating the presence of falsely higher fibrosis stage than the actual fibrosis stage by both TE[7-9] and ARFI[10-12]. However, Rizzo *et al*[13] reported that ARFI is not correlated with alanine aminotransferase (ALT) levels[13].

Liver fibrosis is usually semi-quantitatively assessed by the numerical systems of Scheuer[14], the Metavir group[15] or Ishak *et al*[16]. Direct measurements of the amount of fibrosis in a biopsy specimen by computer-assisted morphometric image analysis has been reported, in which morphometric collagen content is measured quantitatively; it has been shown to correlate well with liver biopsy assessment numerical systems scores[17-19]. Isgro *et al*[20] reported that fibrosis area has a better relationship with TE than Ishak stage[20], whereas our previous study demonstrated a better correlation of TE with fibrosis stage than with fibrosis area in patients with chronic hepatitis C[21].

In the present study, factors other than fibrosis stage that affect ARFI were investigated in patients with chronic hepatitis C. The proportion of fibrosis area was quantitatively measured by image analysis software in liver biopsy specimens and the correlation with ARFI was assessed.

MATERIALS AND METHODS

Ethical statement

This study was performed in strict accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Fujita Health University ethics committee. All study participants provided written informed consent.

Patients

A total of 108 consecutive patients with chronic hepatitis C virus infection who underwent a liver biopsy before treatment with interferon at Fujita Health University Hospital from October 2009 to October 2012 were included (Table 1). Liver biopsy was performed using a 14G disposable true-cut needle under ultrasonographic guidance. Sections were stained with hematoxylin-eosin and azan stain. Liver specimens of at least 1.5 cm length with more than 8 portal tracts were assessed. Liver biopsy specimens were assessed by two hepatologists (Yoshioka K and Nakaoka K) blinded to the clinical data according to Metavir score[15]. Fibrosis was staged as follows: *F*0, no fibrosis; *F*1, portal fibrosis without septa; *F*2, portal fibrosis and few septa; *F*3, numerous septa without cirrhosis; and *F*4, cirrhosis. Activity was graded as follows: A0, none; A1, mild; A2, moderate; and A3, severe activity. Steatosis was graded according to the nonalcoholic fatty liver disease activity score as follows: grade 0; < 5% of hepatocytes involved, grade 1, 5%-33%; grade 2, >33%-66%; and grade 3, > 66%[22]. When fibrosis stage, activity grade, or steatosis grade evaluated by the two hepatologists differed, the higher fibrosis stage, activity grade, or steatosis grade was adopted.

ARFI measurement

Vs measurement by ARFI was performed with a Siemens ACUSON S2000 (Mochida Siemens Medical Systems Co., Ltd., Tokyo, Japan) within 1 wk of liver biopsy[4]. A region in liver to be examined for elastic properties is targeted with a region-of-interest (ROI) cursor while performing B-mode imaging. Tissue at the ROI is mechanically excited using acoustic push pulses to generate localized tissue displacements. The displacements result in propagation of shear-wave away from the region of excitation which is tracked using ultrasonic correlation-based methods. The maximal displacement is estimated for many ultrasound tracking beams laterally adjacent to the single push-beam. By measuring the time to peak displacement at each lateral location, the shear wave propagation velocity can be reconstructed. The examination was performed on the right lobe of the liver. A measurement depth of 2-3 cm below the liver capsule was chosen. Ten successful acquisitions at different locations were performed on each patient, and the results are expressed in meters/second (m/s), and the median value was calculated. The shear wave propagation velocity is considered to be proportional to the square root of tissue elasticity.

The procedures were performed by two investigators (Nishikawa T and Hashimoto S) who were blind to clinical, serological and histological data. The correlation in Vs measurement between two operators was good (r = 0.934).

Proportion of fibrosis area in the liver biopsy specimens

The proportion of fibrosis area in the biopsy specimens was measured by computer-assisted morphometric image analysis. Liver biopsy specimens were stained with azan stain. Microscopic images of the entire biopsy specimen were obtained with a digital microscope (BZ-9000, Keyence, Tokyo, Japan). Fibrosis area, which was stained blue with azan, was marked and measured with Image Pro

Table 1 Characteristics of 108 patients with chronic hepatitis C virus infection

	All patients (n = 108)	F0 (n = 14)	*P* values of Mann-Whitney *U* test between F0 and F1	F1 (n = 17)	*P* values of Mann-Whitney *U* test between F0-1 and F2	F2 (n = 32)	*P* values of Mann-Whitney *U* test between F2 and F3	F3 (n = 31)	*P* values of Mann-Whitney *U* test between F3 and F4	F4 (n = 14)
Age (yr)[1]	59.5 (49.0-66.0)	48.0 (41.0-60.0)	NS	51.0 (41.8-65.5)	NS	61.5 (51.5-66.5)	NS	61.0 (52.0-67.0)	NS	60.5 (54.0-66.0)
Gender (female/male)[2]	52/56	8/6	NS	8/9	NS	15/17	NS	13/18	NS	8/6
BMI	22.5 (20.5-24.6)	22.0 (20.0-23.2)	NS	23.5 (19.8-25.4)	NS	23.0 (20.7-24.4)	NS	23.2 (21.1-26.1)	NS	21.9 (19.0-23.4)
Fibrosis stage (F0/F1/F2/F3/F4)	14/17/32/31/14	-		-		-		-		-
Inflammatory grade (A0/A1/A2/A3)	12/32/53/11	9/5/0/0	0.0261	2/15/0/0	0.0001	1/9/22/0	0.0060	0/2/22/7	NS	0/1/9/4
Steatosis grade (S0/S1/S/2/S3)	42/42/14/10	8/5/0/1	NS	7/9/0/1	NS	10/11/6/5	NS	9/14/5/3	NS	8/3/3/0
AST (IU/L)[1]	44.0 (31.5-82.0)	28.5 (24.0-38.0)	NS	36.0 (23.0-41.3)	0.0033	48.5 (36.5-101.5)	NS	48.0 (42.5-85.3)	NS	65.5 (37.0-88.0)
ALT (IU/L)[1]	55.0 (35.0-91.5)	37.5 (22.0-59.0)	NS	39.0 (24.8-52.0)	0.0095	65.0 (41.0-153.0)	NS	70.0 (41.3-109.0)	NS	64.0 (36.0-91.0)
γ-GTP (IU/L)[1]	33.0 (23.5-75.0)	23.0 (14.0-27.0)	0.0802	28.0 (19.5-71.3)	NS	39.5 (24.5-89.5)	NS	41.0 (28.0-96.8)	0.0329	30.5 (27.0-38.0)
Platelet count ($\times 10^4/\mu L$)[1]	14.3 (11.3-17.6)	14.6 (11.7-20.2)	NS	18.2 (16.6-21.2)	0.0107	16.1 (14.0-17.4)	0.0080	12.2 (11.3-14.3)	0.0078	10.1 (7.1-11.6)
Prothrombin time (INR)[1]	1.00 (0.96-1.06)	0.95 (0.90-0.99)	NS	0.96 (0.93-1.02)	NS	1.00 (0.95-1.03)	0.0144	1.03 (1.00-1.08)	0.0229	1.10 (1.03-1.12)
Albumin (g/dL)[1]	4.2 (4.0-4.5)	4.4 (4.1-4.6)	NS	4.4 (4.2-4.5)	NS	4.3 (4.0-4.5)	0.0524	4.1 (3.8-4.3)	NS	4.0 (3.8-4.2)
Total cholesterol (mg/dL)[1]	170 (150-188)	193 (177-207)	0.0619	169 (155-193)	NS	172 (156-189)	0.0615	159 (141-177)	NS	160 (144-183)
γ-globulin (g/dL)[1]	1.51 (1.33-1.79)	1.28 (1.14-1.40)	0.0262	1.40 (1.28-1.70)	NS	1.44 (1.34-1.64)	0.0067	1.63 (1.51-2.11)	NS	1.66 (1.43-1.97)
Hyaluronic acid (ng/mL)[1]	89 (49-206)	39 (30-64)	NS	49 (26-77)	0.0041	89 (66-185)	0.0601	184 (82-245)	0.0291	232 (191-338)
HCV genotype (1/2)	81/26	10/4	NS	12/5	NS	22/9	NS	5/26	NS	11/3
HCV RNA (logIU/mL)[1]	6.6 (5.8-7.0)	6.5 (6.0-6.9)	NS	6.6 (5.4-7.0)	NS	6.6 (5.8-7.1)	NS	6.7 (5.9-7.1)	NS	6.6 (6.3-6.8)
Fibrosis area (%)[1]	2.63% (1.35-4.95)	0.85% (0.41-1.04)	0.0111	1.37% (0.73-1.85)	0.0022	2.20% (1.62-2.74)	< 0.0001	4.83% (4.03-6.24)	< 0.0001	8.87% (8.04-10.52)
Velocity of shear wave (m/s)[1]	1.38 (1.19-1.71)	1.2 (1.0-1.3)	NS	1.1 (1.0 -1.2)	0.0010	1.3 (1.2-1.6)	0.0014	1.6 (1.5-1.8)	0.0008	2.1 (1.9-2.2)

[1]Data are shown as median (interquartile range); [2]Difference of frequency of gender was assessed by χ^2 test. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyltranspeptidase.

Plus 4.0 imaging software (Nippon Roper Co., Ltd., Tokyo, Japan).

Statistical analysis

Patients were categorized according to fibrosis stage. The groups were compared with the χ^2 test and Mann-Whitney *U* test. Factors correlated with ARFI were estimated by Spearman's rank correlation test. Factors independently correlated with ARFI were assessed by multiple regression analysis. The diagnostic performance of ARFI and fibrosis area was determined in terms of sensitivity, specificity, positive and negative predictive value, positive likelihood ratio, diagnostic accuracy, and area under the receiver operating characteristics (ROC) curve. Optimal cutoff values between fibrosis categories were determined at maximum sum of sensitivity and specificity. Data were analyzed using StatFlex version 5.0 for Windows (StatFlex, Osaka, Japan). A two-sided *P* value of < 0.05 indicated statistical significance.

RESULTS

Semiquantitative histological assessment using the Metavir system

The liver biopsies of the 108 patients were assessed by the Metavir system. Fibrosis stage was *F*0 in 14 patients, *F*1 in 17, *F*2 in 32, *F*3 in 31 and *F*4 in 14 (Table 1).

ARFI measurement

ARFI was significantly correlated with fibrosis stage ($\rho = 0.732$, $P < 0.0001$) (Figure 1A). ARFI values differed significantly between stages *F*1 and *F*2 ($P = 0.0010$), between *F*2

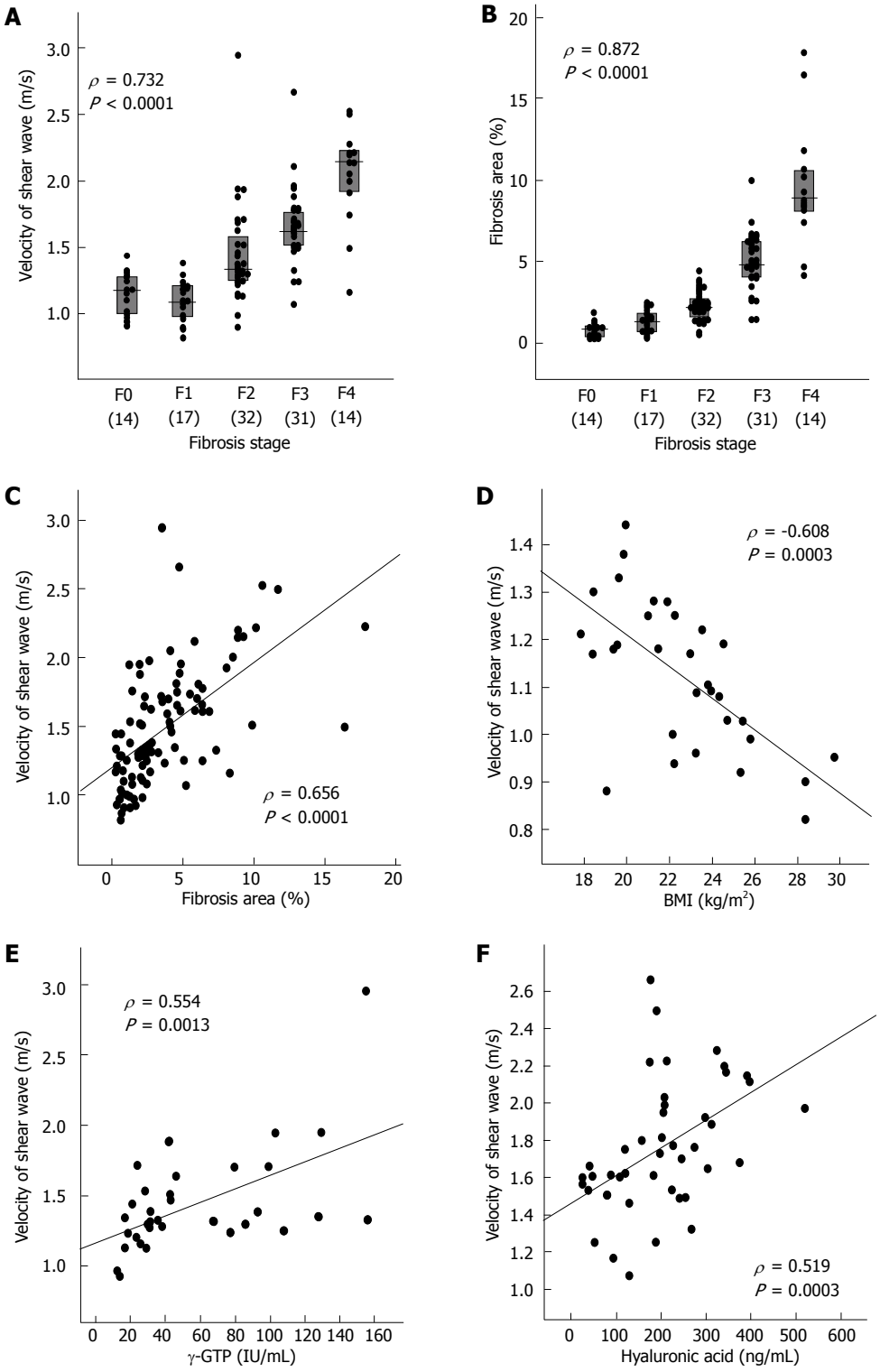

Figure 1 Correlation. A: Between acoustic radiation force impulse (ARFI) and fibrosis stages. The velocity of the sheer wave measured by ARFI was significantly correlated with fibrosis stage in all 108 patients as assessed by the Metavir system (ρ = 0.732, P < 0.0001). Vertical lines and boxes indicate median values and interquartile ranges, respectively; B: Between proportion of fibrosis area and fibrosis stage. The proportion of fibrosis area was significantly correlated with fibrosis stage in all 108 patients as assessed by the Metavir system (ρ = 0.872, P < 0.0001). Vertical lines and boxes indicate median values and interquartile ranges, respectively; C: Between ARFI and proportion of fibrosis area. The velocity of the sheer wave measured by ARFI significantly correlated with the proportion of fibrosis area in all 108 patients (ρ = 0.656, P < 0.0001); D: Between ARFI and body mass index (BMI). The velocity of the sheer wave measured by ARFI significantly negatively correlated with BMI in patients with stage F0 or F1 (ρ = −0.608, P = 0.0003); E: Between ARFI and γ-glutamyltranspeptidase (γ-GTP) levels. The velocity of the sheer wave measured by ARFI significantly correlated with γ-GTP levels in patients with stage F2 (ρ = 0.544, P = 0.0013); F: Between ARFI and hyaluronic acid levels. The velocity of the sheer wave as measured by ARFI significantly correlated with hyaluronic acid levels in patients with stage F3 or F4 (ρ = 0.519, P = 0.0003).

and *F*3 (P = 0.0014), and between *F*3 and *F*4 (P = 0.0008) (Table 1).

The ARFI cutoff values for different fibrosis stages determined by ROC analysis were 1.28 m/s for $F \geqslant 1$,

Table 2 Optimal cutoff value of velocity of shear wave for each fibrosis stage was determined at maximum sum of sensitivity and specificity

	$F \geq 1$	$F \geq 2$	$F \geq 3$	$F4$
Cutoff value (m/s)	1.28	1.28	1.44	1.73
Positive predictive value	97.0%	94.0%	78.4%	48.0%
Negative predictive value	29.3%	65.9%	91.2%	97.6%
Sensitivity	69.1%	81.8%	88.9%	85.7%
Specificity	85.7%	87.1%	82.5%	86.2%
Positive likelihood ratio	4.8	6.3	5.1	6.2
Diagnostic accuracy	71.3%	83.3%	85.2%	86.1%
AUROC	0.810	0.909	0.869	0.885
Standard error of AUROC	0.046	0.027	0.036	0.058

AUROC: Area under receiver operating characteristic curve

Table 3 Optimal cutoff value of fibrosis area for each fibrosis stage was determined at maximum sum of sensitivity and specificity

	$F \geq 1$	$F \geq 2$	$F \geq 3$	$F4$
Cutoff value	1.17%	1.8%	3.71%	7.32%
Positive predictive value	97.7%	94.3%	93.0%	92.3%
Negative predictive value	60.0%	71.1%	92.3%	97.9%
Sensitivity	91.5%	85.7%	88.9%	85.7%
Specificity	85.7%	87.1%	95.2%	98.9%
Positive likelihood ratio	6.4	6.6	18.7	80.6
Diagnostic accuracy	90.7%	86.1%	92.6%	97.20%
AUROC	0.935	0.927	0.963	0.962
Standard error of AUROC	0.025	0.024	0.018	0.023

AUROC: Area under receiver operating characteristic curve

1.28 m/s for $F \geq 2$, 1.44 m/s for $F \geq 3$, and 1.73 m/s for *F*4 (Table 2).

Fibrosis area in liver biopsy specimens

The proportion of fibrosis area was significantly correlated with fibrosis stage as assessed by the Metavir system ($\rho = 0.872$, $P < 0.0001$) (Figure 1B). The fibrosis area values differed significantly between stages *F*0 and *F*1 ($P = 0.0111$), *F*1 and *F*2 ($P = 0.0022$), *F*2 and *F*3 ($P < 0.0001$), and between *F*3 and *F*4 ($P < 0.0001$) (Table 1).

The fibrosis area cutoff values for the different fibrosis stages determined by ROC analysis were 1.17% for $F \geq 1$, 1.80% for $F \geq 2$, 3.71% for $F \geq 3$, and 7.32% for *F*4 (Table 3).

Factors correlating with ARFI in all 108 patients

ARFI was significantly correlated with fibrosis stage ($P < 0.0001$) (Figure 1A), inflammatory grade ($P < 0.0001$), aspartate aminotransferase (AST) levels ($P < 0.0001$), ALT levels ($P = 0.0008$), γ-glutamyltranspeptidase (γ-GTP) levels ($P < 0.0001$), platelet count ($P < 0.0001$), prothrombin time (INR) ($P = 0.0003$), albumin levels ($P = 0.0002$), total cholesterol levels ($P = 0.0004$), γ-globulin levels ($P = 0.0087$), hyaluronic acid levels ($P < 0.0001$), and fibrosis area ($P < 0.0001$) (Figure 1) by Spearman's rank correlation test (Table 4). ARFI tended to be higher in genotype 1 [median, 1.49 (interquartile range, 1.22-1.75) m/s] than in genotype 2 [1.30 (1.17-1.46)] ($P = 0.0728$). The multiple regression analysis selected fibrosis stage ($\beta = 0.1865$, $P < 0.0001$) and hyaluronic acid levels ($\beta = 0.0008$, $P = 0.0039$) as factors that independently correlated with ARFI, whereas inflammatory grade, AST, ALT and fibrosis area were not selected (Table 4).

Factors correlating with ARFI in stage F0 or F1 patients

To elucidate the factors affecting ARFI other than fibrosis stage, patients with stage *F*0 or *F*1, those with *F*2, and those with *F*3 or *F*4 were analyzed separately.

Body mass index (BMI) was significantly correlated with ARFI ($P = 0.0003$) (Figure 1D) and ALT levels ($P = 0.0593$) and γ-GTP levels ($P = 0.0614$) tended to be correlated with ARFI by Spearman's rank correlation test in the 31 patients with stage *F*0 or *F*1 (Table 4). Only BMI was correlated with ARFI by multiple regression analysis ($\beta = -0.0334$, $P = 0.0001$).

Factors correlating with ARFI in the stage F2 patients

γ-GTP levels were significantly correlated with ARFI ($P = 0.0013$) (Figure 1E) and γ-globulin levels ($P = 0.0581$) tended to be correlated with ARFI in the 32 patients with stage *F*2 by Spearman's rank correlation test (Table 4). The multiple regression analysis only selected γ-GTP levels as a factor correlating with ARFI ($\beta = 0.0048$, $P = 0.0012$).

Factors correlating with ARFI in the stage F3 or F4 patients

In the patients with stage *F*3 or *F*4, fibrosis stage ($P = 0.0004$), platelet count ($P = 0.0036$), prothrombin time (INR) ($P = 0.0080$), albumin levels ($P = 0.0015$), hyaluronic acid levels ($P = 0.0003$) (Figure 1F), and fibrosis area ($P = 0.0481$) were significantly correlated with ARFI by Spearman's rank correlation test (Table 4). The multiple regression analysis selected fibrosis stage ($\beta = 0.2921$, $P = 0.0044$) and hyaluronic acid levels ($\beta = 0.0012$, $P = 0.0025$) as factors correlating with ARFI.

DISCUSSION

The assessment of fibrosis stage is important to estimate prognosis and to identify the patients requiring antiviral treatment in chronic hepatitis C. A lot of noninvasive methods to assess liver fibrosis stage other than liver biopsy are available, for example, ARFI, TE, real-time elastography[23], and algorithm of serum fibrosis markers such as FibroTest[24] and APRI[25]. They provide good performances in estimation of fibrosis stage, while there are problems such as influence of inflammation. In the present study, factors other than fibrosis stage that affect ARFI were investigated in patients with chronic hepatitis C.

The present study confirmed findings reported previously that ARFI correlates with fibrosis stage[10-13,26,27]. The ARFI cutoff values for different fibrosis stages were 1.28 m/s for $F \geq 1$, 1.28 m/s for $F \geq 2$, 1.44 m/s for $F \geq 3$ and 1.73 m/s for *F*4. This result suggests that distinguishing between *F*0 and *F*1 is impossible, as the cutoff

Table 4 Factors correlating with velocity of shear wave in 108 patients with chronic hepatitis C virus infection

	All patients (n = 108)				Patients with $F0$ or $F1$ (n = 31)				Patients with $F2$ (n = 32)				Patients with $F3$ or $F4$ (n = 45)			
	Spearman's rank correlation test		Multiple regression analysis		Spearman's rank correlation test		Multiple regression analysis		Spearman's rank correlation test		Multiple regression analysis		Spearman's rank correlation test		Multiple regression analysis	
	ρ	*P* value	β	*P* value	ρ	*P* value	β	*P* value	ρ	*P* value	β	*P* value	ρ	*P* value	β	*P* value
Age (yr)		NS				NS				NS				NS		
Gender (female/male)[1]		NS				NS				NS				NS		
BMI		NS			-0.608	0.0003	-0.033	0.0001		NS				NS		
Fibrosis stage	0.732	< 0.0001	0.187	0.0001		NS							0.505	0.0004	0.292	0.0044
Inflammatory grade	0.612	< 0.0001		NS		NS				NS				NS		
Steatosis grade		NS				NS				NS				NS		
AST (IU/L)	0.430	< 0.0001		NS		NS				NS				NS		
ALT (IU/L)	0.318	0.0008		NS	0.343	0.0593		NS		NS				NS		
γ-GTP (IU/L)	0.407	< 0.0001		NS	0.340	0.0614		NS	0.544	0.0013	0.005	0.0012		NS		
Platelet count ($\times 10^4$/μL)	-0.441	< 0.0001		NS		NS				NS			-0.425	0.0036		NS
Prothrombin time (INR)	0.344	0.0003		NS		NS				NS			0.390	0.0080		NS
Albumin (g/dL)	-0.347	0.0002		NS		NS				NS			-0.459	0.0015		NS
Total cholesterol (mg/mL)	-0.337	0.0004		NS		NS				NS				NS		
γ-globulin (g/dL)	0.252	0.0087		NS		NS			-0.344	0.0581		NS		NS		
Hyaluronic acid (ng/mL)	0.576	< 0.0001	8.00E-4	0.0039		NS				NS			0.519	0.0003	0.001	0.0025
HCV genotype (1/2)[1]		0.0728		NS		NS				NS				NS		
HCV RNA (logIU/mL)		NS				NS				NS				NS		
Fibrosis area (%)	0.656	< 0.0001		NS		NS				NS			0.296	0.0481		NS
R			0.707				0.645				0.546				0.634	
Adjusted R			0.490				0.396				0.275				0.373	
F			51.800				20.700				12.700				14.100	
P value			< 0.0001				0.0001				0.0012				< 0.0001	

[1]Difference of frequency of gender or genotype was assessed by Mann-Whitney *U* test. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyltranspeptidase.

value for $F \geqslant 1$ and that for $F \geqslant 2$ are the same. However, Sporea *et al*[26] reported that the cutoff value is 1.19 m/s for $F \geqslant 1$, 1.33 m/s for $F \geqslant 2$, 1.43 m/s for $F \geqslant 3$, and 1.55 m/s for $F4$[26]. Rizzo *et al*[13] reported that the cutoff value is 1.3 m/s for $F \geqslant 2$, 1.7 m/s for $F \geqslant 3$ and 2.0 m/s for $F4$[13]. Thus, discrepancies are apparent among the cutoff values reported in different studies. The discrepancies are probably attributed to the difference in the population studied. Further studies should be conducted to establish standard ARFI cutoff values for staging fibrosis.

In the present study, AST, ALT and inflammatory grade were correlated with ARFI in the univariate analysis that included all patients, but were not selected as factors independently correlating with ARFI in the multiple regression analysis. In addition, inflammatory factors did not correlate with ARFI when patients with different fibrosis stages were analyzed separately. These results suggest that inflammatory activity does not affect ARFI in patients with chronic hepatitis C. Rizzo *et al*[13] also reported that ARFI is not associated with ALT, BMI, Metavir grade, or liver steatosis, whereas TE is significantly correlated with ALT[13]. Bota *et al*[10] reported that discordance of at least two fibrosis stages between ARFI and histologic assessment were associated with female sex, interquartile range interval (IQR) $\geqslant$ 30%, high AST and high ALT in univariate analysis, while, in multivariate analysis, the female gender and IQR $\geqslant$ 30% ($P = 0.004$) were associated with the discordances. In contrast, Yoon *et al*[12] reported that the optimum ARFI cutoff values are 1.13 m/s for $F \geqslant 2$ and 1.98 m/s for $F4$, whereas these values decreased to 1.09 m/s for $F \geqslant 2$ and 1.81 m/s for $F4$ when patients with normal ALT levels were selected. Chen *et al*[11] reported that ALT, ActiTest A score, Metavir activity (A) grade, Metavir F stage, BMI, and platelet count are independently associated with ARFI and suggested that a 100 IU/L increase in serum ALT levels augmented ARFI by approximately 0.155 m/s. In the present study, only 25 patients had ALT levels of 100 IU/L

or higher. The low ALT levels among the patients studied may be a reason why ALT was not correlated with ARFI.

A multiple linear regression analysis in our previous study on TE selected fibrosis area, ALT levels, γ-GTP levels, prothrombin time, and hyaluronic acid levels as factors correlating with TE[21]. Many studies on TE have reported that LSM is affected by ALT levels. Franquelli *et al*[28] reported that TE fibrosis staging is overestimated by necroinflammatory activity and steatosis. Coco *et al*[7] found that LSM is higher in patients with an elevated ALT than in those with either spontaneous biochemical remission or after antiviral therapy. Thus, it is probable that ALT or inflammatory activity affects TE. However, it is still unclear whether they also affect ARFI. Further studies are needed to clarify factors that affect ARFI other than fibrosis stage.

ARFI was significantly correlated with BMI in the 31 patients with stage *F*0 or *F*1; the higher the BMI, the lower the ARFI. However, ARFI was not associated with steatosis grade. Motosugi *et al*[29] reported that fat deposition in the liver does not affect ARFI. Thus, the negative correlation between BMI and ARFI could not be attributed to steatosis, which accompanies higher BMI[30]. Actually, BMI and steatosis grade were not correlated in patients with stage *F*0 or *F*1 in the present study (data not shown). The mechanism of the association between higher BMI and lower ARFI is unclear. Because a higher BMI is associated with lower ARFI, and may cause an underestimation of fibrosis staging, careful attention should be paid to BMI during ARFI staging of fibrosis in patients with stage *F*0 or *F*1 disease.

ARFI significantly correlated with γ-GTP levels in patients with *F*2 and with fibrosis stage and hyaluronic acid levels in patients with stage *F*3 or *F*4. γ-GTP[24,31] and hyaluronic acid[32,33] levels have been regarded as the most informative fibrosis markers. Thus, it is reasonable that γ-GTP and hyaluronic acid levels independently correlated with ARFI.

Isgro *et al*[20] showed that the collagen proportional area has a better relationship with TE and with hepatic venous pressure gradient compared with Ishak stage. In the present study, fibrosis area was correlated significantly with fibrosis stage, but only fibrosis stage and hyaluronic acid levels were selected as factors independently correlating with ARFI. Our previous study demonstrated a better correlation of TE with fibrosis stage than with fibrosis area in patients with chronic hepatitis C[21]. The Metavir stages represent categories of increasing fibrosis severity based on a combination of location and quantity of scarring as well as whether the fibrous tissue forms septa, bridges, or nodules. Fibrosis area represents only the quantity of fibrosis in liver tissues. Our results indicate that not only the quantity of fibrosis but also other histological factors such as patterns of fibrosis also affect ARFI.

The present study demonstrated that ARFI correlated with fibrosis stage but was not associated with inflammation. BMI negatively correlated with ARFI in the patients with stage *F*0 or *F*1. γ-GTP and hyaluronic acid levels were positively correlated in those with stage *F*2 and in those with *F*3 or *F*4, respectively. Thus, careful attention should be paid to BMI, γ-GTP levels, and hyaluronic acid levels when estimating fibrosis stage by ARFI. Fibrosis stage showed a better correlation with ARFI than fibrosis area, indicating that not only the quantity of fibrosis but also other factors such as patterns of fibrosis also affect ARFI. Since the number of the patients studied is small, further studies are needed to confirm the conclusion of the present study.

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COMMENTS

Background

Most studies reported that liver stiffness measurement by Fibroscan was affected by inflammation. There have been both of the reports which demonstrated the correlation of inflammation and acoustic radiation force impulse (ARFI) and those which denied their correlation. The present study confirmed findings reported previously that ARFI correlates with fibrosis stage, and demonstrated that aspartate aminotransferase, alanine aminotransferase and inflammatory grade did not independently correlate with ARFI in the multiple regression analysis. The present study also demonstrated the correlation of body mass index (BMI) and ARFI for the first time.

Innovations and breakthroughs

The new findings of this study are the correlation of BMI and ARFI, and the denial of the correlation between ARFI and inflammation.

Applications

The results showed that ARFI correlated significantly with liver fibrosis stage and hyaluronic acid in all patients. ARFI correlated significantly with BMI in fibrosis stage F0-1, with γ-glutamyltranspeptidase (GTP) in F2, and with fibrosis stage and hyaluronic acid in F3-4. In conclusion, ARFI correlated with fibrosis stage and hyaluronic acid but not with inflammation. ARFI was affected by BMI, γ-GTP, and hyaluronic acid in each fibrosis stage.

Peer review

The authors reported the utilities of ARFI elastography for evaluation of hepatic fibrosis in patients with chronic hepatitis C. This paper looks very important and has a novelty in this study field.

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BRIEF ARTICLE

Antinociceptive effects of novel melatonin receptor agonists in mouse models of abdominal pain

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Abstract

AIM: To characterize the antinociceptive action of the novel melatonin receptor (MT) agonists, Neu-P11 and Neu-P12 in animal models of visceral pain.

METHODS: Visceral pain was induced by intracolonic (*ic*) application of mustard oil or capsaicin solution or by intraperitoneal (*ip*) administration of acetic acid. Neu-P11, Neu-P12, or melatonin were given *ip* or orally and their effects on pain-induced behavioral responses were evaluated. To identify the receptors involved, the non-selective MT1/MT2 receptor antagonist luzindole, the MT2 receptor antagonist 4-P-PDOT, or the μ-opioid receptor antagonist naloxone were injected *ip* or intracerebroventricularly (*icv*) prior to the induction of pain.

RESULTS: Orally and *ip* administered melatonin, Neu-P11, and Neu-P12 reduced pain responses in a dose-dependent manner. Neu-P12 was more effective and displayed longer duration of action compared to melatonin. The antinociceptive effects of Neu-P11 or Neu-P12 were antagonized by *ip* or *icv*. administered naloxone. Intracerebroventricularly, but not *ip* administration of luzindole or 4-P-PDOT blocked the antinociceptive actions of Neu-P11 or Neu-P12.

CONCLUSION: Neu-P12 produced the most potent and long-lasting antinociceptive effect. Further development of Neu-P12 for future treatment of abdominal pain seems promising.

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Key words: Gastrointestinal tract; Melatonin; Neu-P11; Neu-P12; Opioid; Visceral pain

Core tip: In search for new efficient therapies for the treatment of pain in the irritable bowel syndrome, the antinociceptive activity of two novel melatonin receptor agonists, Neu-P11 and Neu-P12, was characterized in a well-established mouse model of visceral pain. Neu-P12 produced a potent and long-lasting antinociceptive effect after intraperitoneal and oral administration. Further development of this novel compound for future treatment of abdominal pain seems promising.

Chen C, Fichna J, Laudon M, Storr M. Antinociceptive effects of novel melatonin receptor agonists in mouse models of abdominal pain. *World J Gastroenterol* 2014; 20(5): 1298-1304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1298.

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INTRODUCTION

Melatonin is a hormone synthesized primarily in the pineal gland and in peripheral organs, including the gastrointestinal tract, bone marrow, and blood cells[1-3]. Following synthesis, melatonin is released into the blood stream and acts as an endocrine hormone controlling biological functions with circadian rhythms, like the sleep-wake cycle. It is a lipophilic compound diffusing rapidly through biological membranes and is amongst others involved in the regulation of intestinal reflexes, metabolism, and reproduction[4]. In the pineal gland melatonin is synthesized and secreted in a circadian manner, with secretion being highest during nighttime[5].

Morris and Lutsch[6] showed that darkness and elevated levels of melatonin decrease sensitivity to pain, suggesting that the hormone may play a significant role in the modulation of pain. In the following years, several studies characterized the antinociceptive effect of melatonin in multiple animal models[7]. Melatonin receptors (MT) mediate or modulate antinociceptive effects at spinal and supraspinal levels[8]. More recently, melatonin-induced antinociception has been reported using neuropathic pain models[9].

Recent clinical trials provided evidence for a beneficial role of melatonin in gastrointestinal functional pain[10]. Double-blinded placebo-controlled clinical trials showed that melatonin reduced extra-colonic symptoms and abdominal pain in patients with irritable bowel syndrome (IBS)[11-14].

Although melatonin has been studied for the treatment of many diseases such as cancer, cardiovascular diseases, depression, seasonal affective disorder, circadian rhythm sleep disorders and insomnia, its actions are limited by rapid degradation and short half-life. Recently, two novel melatonin receptor agonists with high affinity at MT receptors and prolonged duration of action, Neu-P11 and Neu-P12, were developed[15-17]. It was shown that Neu-P11 and Neu-P12 display long half-life and oral availability and are thus promising drug candidates, which require further characterization.

In the present study, we evaluated the possible antinociceptive effects of Neu-P11 and Neu-P12 in mouse models of visceral pain and compared their effects to those of melatonin. We also aimed at characterizing the mechanism of action of Neu-P11 and Neu-P12 *via* identifying the receptors involved.

MATERIALS AND METHODS

Animals

Male Swiss albino CD1 mice, weighing 25-30 g were obtained from Charles River (Canada). Animals were housed at a constant temperature of 22 ℃ and kept at a constant photoperiod (12:12-h light-dark cycle) in saw-dust-lined plastic cages with free access to standard laboratory chow and tap water. The animal use for these studies was approved by the University of Calgary Animal Care Committee and the experiments were performed in accordance with institutional animal ethics committee guidelines that are in agreement to the guidelines established by the Canadian Council on Animal Care.

Behavioral responses to mustard oil and capsaicin solution

Behavioral pain-related responses to intracolonic (*ic*) administration of mustard oil (MO) and capsaicin solution were determined in the morning as described previously[18-20]. Fifteen minutes after *ip* injection of Neu-P11, Neu-P12, or melatonin (25 and 50 mg/kg) or 20 min after oral gavage (25, 50 and 100 mg/kg), 50 μL of MO (1% vol/vol dissolved in 70% ethanol) or capsaicin (0.3% w/v in 10% ethanol, 10% Tween 80, 80% saline) were administered into the colon of anesthetized mice using a fine catheter (external diameter 0.61 mm, 4 cm long, Minipack, Portex, Hythe, United Kingdom). Vaseline was applied to the perianal area to avoid the stimulation of somatic areas by contact with MO or capsaicin. After the administration of MO or capsaicin, the animals were placed in individual plastic cages in a quiet environment. Five minutes later, spontaneous pain-related responses: licking of the abdomen, stretching the abdomen, squashing the lower abdomen against the floor, and abdominal retractions were counted for 20 min.

Typically, the number of behaviors in MO- and capsaicin-treated mice was 60-70 and 30-40, respectively. In these conditions even slight changes in the number of behaviors, caused by either anti- or pro-nociceptive action of studied compounds, can be noticed, but simultaneously only statistically significant differences in obtained values reflect pharmacologically relevant action.

The non-selective MT1/MT2 receptor antagonist luzindole (5 mg/kg), the selective MT2 receptor antagonist 4-P-PDOT (4 mg/kg) and the opioid receptor antagonist naloxone (1 mg/kg) were administered *ip* 15 min prior to Neu-P11, Neu-P12 and melatonin. To study the role of MT and opioid receptors in the central nervous system, luzindole (5 μg/animal), 4-P-PDOT (10 μg/animal) or naloxone (5 μg/animal) were injected *icv*. Five minutes prior to the *ip* administration of Neu-P11, Neu-P12 and melatonin. Vehicles only were used in control experiments.

Acetic acid induced pain

The acetic acid test was performed as described recently[19-21]. Fifteen minutes after *ip* injection of Neu-P11, Neu-P12, or melatonin (1 and 5 mg/kg), mice received an *ip* injection of an acetic acid solution (0.5%, vol/vol in 0.9% NaCl). Animals were then placed individually in empty cages and after 5 min abdominal stretchings were counted for 15 min in 5 min intervals. A typical stretch was characterized by an elongation of the body and the development of tension in the abdominal muscles and

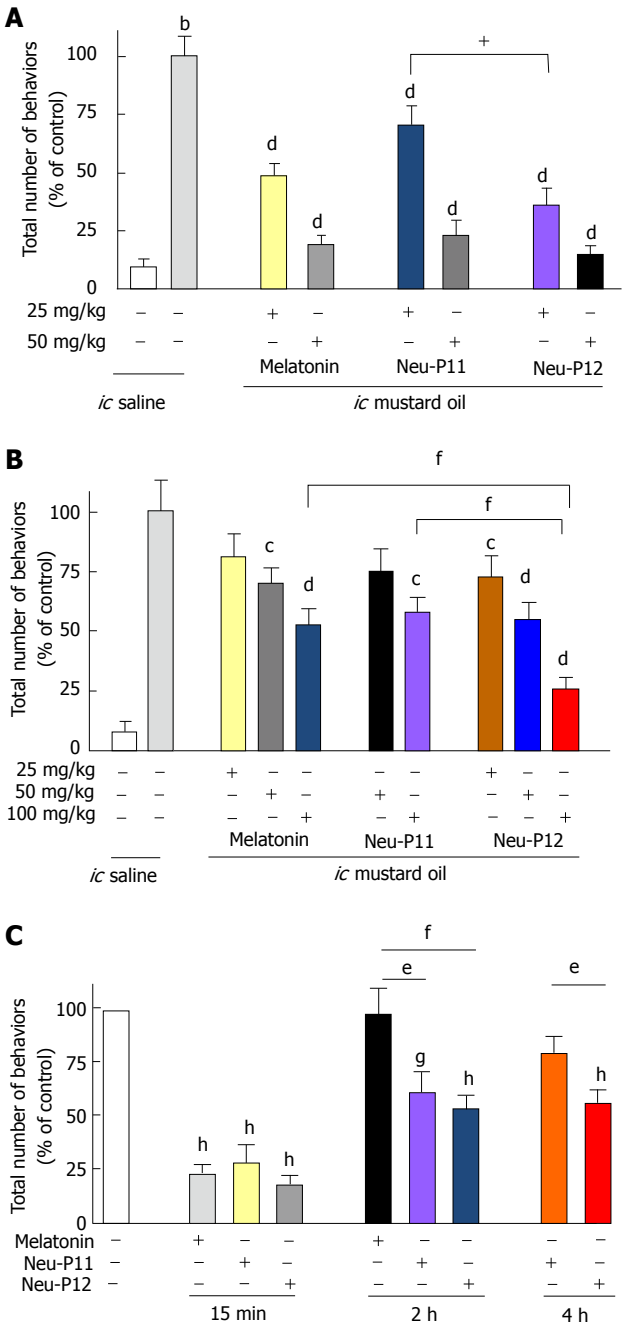

Figure 1 Effects of melatonin and melatonergic agonists on mustard oil-induced pain. A: *Ip* melatonin, Neu-P11, and Neu-P12 (all 25 and 50 mg/kg) reduced the number of pain-related behaviors in the mustard oil (MO) sensitivity test 15 min after drug administration; B: Oral melatonin, Neu-P11 and Neu-P12 (all 25 mg/kg, 50 mg/kg and 100 mg/kg) decreased the number of pain-related behaviors in the MO sensitivity test 20 min after drug administration; C: Effect of ip injected melatonin, Neu-P11 and Neu-P12 (all at 50 mg/kg) on the number of pain-related behaviors in the MO sensitivity test at 15 min, 2 and 4 h after drug administration. Data are mean ± SE (n = 6-10). $^{b}P < 0.01$ *vs* MO; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* vehicle + MO; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* drug; $^{g}P < 0.05$, $^{h}P < 0.01$ *vs* control.

hind paws. Vehicle was used in control experiments. Typically, the number of behaviors in acetic acid solution-treated mice was 30-40.

Drugs

Melatonin, luzindole, 4-P-PDOT, naloxone and capsaicin were obtained from Tocris Bioscience (Tocris, Ellisville, Missouri, United States). Allyl isothiocyanate (mustard oil, MO) was purchased from *Merck* (*Darmstadt*, Germany). Neu-P11 and Neu-P12 were obtained from Neurim Pharmaceuticals Ltd., Israel. All drugs were dissolved in dimethyl sulfoxide and diluted in 0.9% saline to final concentrations.

Statistical analysis

PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, United States) was used for statistical and curve-fitting analyses. One-way analysis of variance followed by Student-Newman-Keuls *post hoc* test was used for analysis of multiple treatment means. P values < 0.05 were considered significant. The data are expressed as mean ± SE.

RESULTS

Effects of melatonin and melatonergic agonists on MO-induced pain

As shown in Figure 1, melatonin, Neu-P11 and Neu-P12 injected *ip* (Figure 1A) or given orally (Figure 1B) significantly reduced the number of pain-related behaviors in the OM visceral pain sensitivity test compared to control in a dose-depended fashion. Oral Neu-P12 was the most effective compound of all melatonin agonists in decreasing the pain behaviors.

The antinociceptive effect of Neu-P12 (*ip* 50 mg/kg) was observed up to 4 h after administration, the effect of Neu-P11 (*ip* 50 mg/kg) up to 2 h. Melatonin (*ip* 50 mg/kg) produced a short-lasting effect, which was observed at 15 min but disappeared at 2 h (Figure 1C).

Effect of naloxone and melatonergic antagonists on melatonin, Neu-P11 and Neu-P12-induced effects on MO-induced pain

The effects of melatonin, Neu-P11 and Neu-P12 on the visceral pain-related behaviors in the MO sensitivity test were blocked by the *ip* administration of the opioid receptor antagonist naloxone, but not by *ip* application of the MT1/2 receptor antagonist luzindole or the MT2 antagonist 4-P-PDOT (Figure 2A). Naloxone and MT receptor antagonists administered directly to the central nervous system all effectively blocked behavioral responses in the visceral pain tests (Figure 2B).

Effect of melatonin, Neu-P11 and Neu-P12 on capsaicin-induced pain

Melatonin, Neu-P11 and Neu-P12 administered *ip* (25 mg/kg) significantly decreased the number of pain induced behaviors following *ic* administration of capsaicin (Figure 3A). Of all compounds tested, Neu-P12 displayed the most potent antinociceptive action.

Effect of melatonin, Neu-P11 and Neu-P12 on the acetic acid induced pain

Melatonin, Neu-P11 and Neu-P12 (5 mg/kg, *ip*) all effectively reduced the total number of visceral pain be-

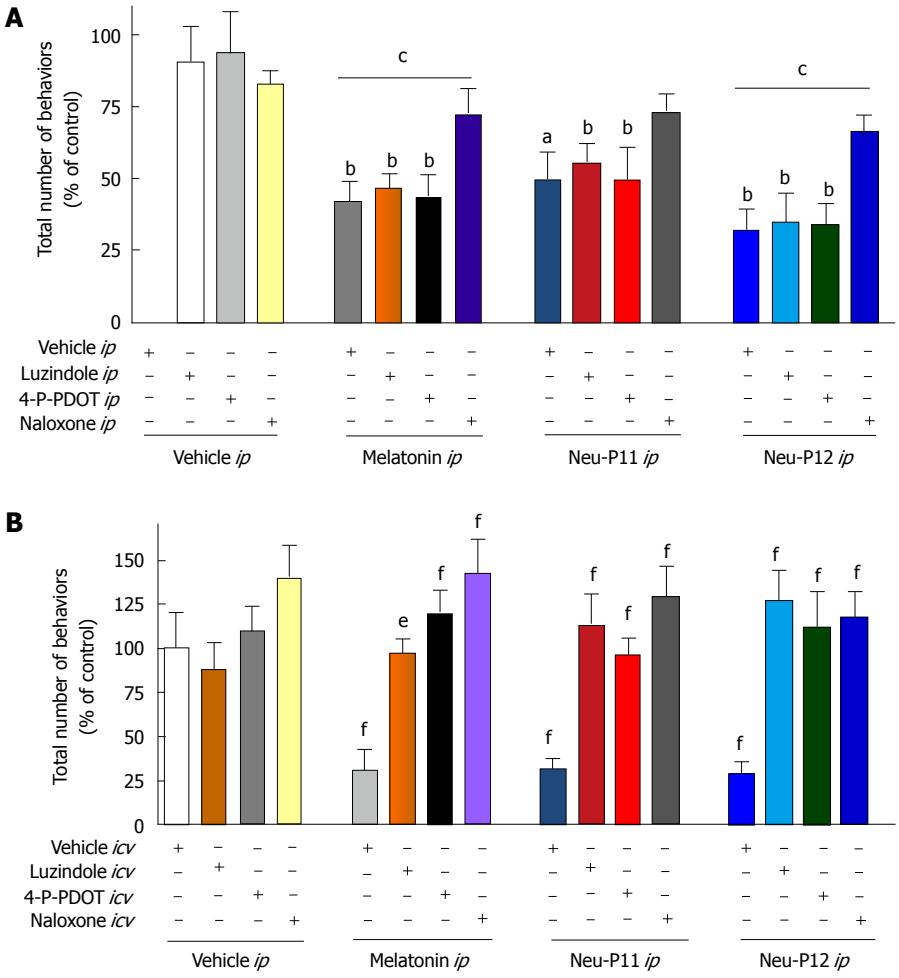

Figure 2 Effect of naloxone and melatonergic antagonists on melatonin, Neu-P11 and Neu-P12-induced effects on mustard oil-induced pain. A: Effects of antagonists: *ip* naloxone (1 mg/kg), but not luzindole (5 mg/kg) or 4-P-PDOT (4 mg/kg) blocked the effects of melatonin, Neu-P11, and Neu-P12 (all at 25 mg/kg, *ip*) on the number of pain-related behaviors in the mustard oil sensitivity test 15 min after drug administration; B: *Icv* naloxone (5 μg/animal), luzindole (5 μg/animal) and 4-P-PDOT (10 μg/animal) blocked the effects of melatonin, Neu-P11 and Neu-P12 (all at 25 mg/kg, *ip*) in the mustard oil sensitivity test 15 min after drug administration. Data are mean ± SE (n = 6-10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$ *vs* drugs + vehicle; $^{e}P < 0.05$, $^{f}P < 0.01$ *vs* vehicle *icv* + drugs *ip*.

haviors induced by the *ip* injection of acetic acid (Figure 3B). Neu-P12 displayed the most potent antinociceptive effect.

DISCUSSION

In this study we showed that *ip* and oral administration of melatonin, Neu-P11 and Neu-P12 produced a potent antinociceptive effect in different animal models of visceral pain of abdominal origin. The analgesic action of all compounds was mediated by central and peripheral opioid and central, but not peripheral, MT receptors.

Melatonin significantly and dose-dependently decreased the total number of pain-related behaviors induced by OM, capsaicin and acetic acid solution in mice. This observation is in line with earlier reports suggesting a role of melatonin and MT receptors in pain signaling. It was shown by others that intrathecal co-administration of low doses of melatonin and morphine attenuated mechanical and thermal hyperalgesia in acute postoperative pain model in rats, suggesting that melatonin acts as a neuromodulator in the spinal cord[22].

In our studies, the two novel non-selective MT1/MT2 melatonergic agonists used, Neu-P11 and Neu-P12, produced a potent antinociceptive effect comparable to that of melatonin and this effect holds true in three different models of visceral pain. The in vivo experiments indicated that Neu-P12 is the most effective drug with the longest duration of action.

We showed that the antinociceptive effects of melatonin, Neu-P11 and Neu-P12 were blocked by the *icv* administration of a MT2 receptor antagonist 4-P-PDOT, as well as *icv* and *ip* injection of naloxone, suggesting a crucial role of central MT2 and opioid receptors, as well peripheral opioid receptors. The involvement of MT2 and opioid receptors in the antinociceptive effects of melatonin was earlier suggested by Ambriz-Tututi and Granados-Soto in models studying other forms of pain, who showed that intrathecal luzindole and 4-P-PDOT and intrathecal or subcutaneous naltrexone blocked the effect of melatonin on tactile allodynia in neuropathic rats[23]. Another study revealed that the antihyperalgesic effect of melatonin on nociceptin-induced hyperalgesia in mice was significantly antagonized by the *icv*. admin-

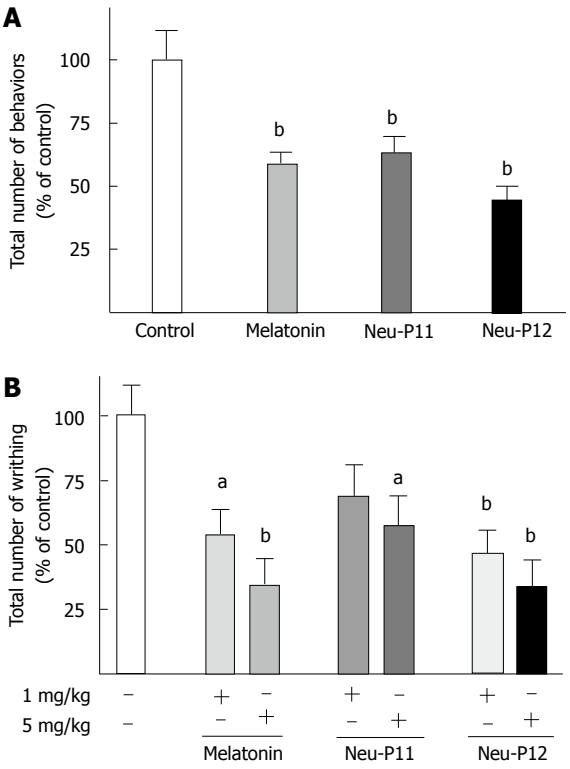

Figure 3 Melatonin, Neu-P11 and Neu-P12 decreased the number of pain-related behaviors. A: Melatonin, Neu-P11 and Neu-P12 (all at 25 mg/kg, *ip*) decreased the number of pain-related behaviors in the capsaicin-induced visceral pain test 15 min after drug administration; B: Melatonin, Neu-P11 and Neu-P12 (all 5 at mg/kg, *ip*) decreased the number of pain-related behaviors in the writhing test 15 min after drug administration. Data are mean ± SE (n = 6-10). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

istration of luzindole or naloxone[24]. Similar results were recently reported by Mickle *et al*[25] suggesting that melatonin attenuates post-inflammatory hypersensitivity through a supra-spinal process linked to the central opioidergic system.

The involvement of opioid receptors in the antinociceptive effects of melatonin and melatonergic agonists observed in our study is an interesting, and complex phenomenon. Earlier receptor binding experiments confirmed the presence of mu- and delta-, but not kappa-opioid bind sites in bovine pinealocyte membranes[26]. (D-Ala^2, N-MePhe^4, Gly-ol)-enkephalin and (D-Pen^2, D-Pen^5)-enkephalin, which are mu- and delta-selective opioid receptor agonists, respectively, enhanced melatonin synthesis in pineal cultures by a naloxone-reversible manner[27,28] and acute administration of morphine induced melatonin release from rat pineal glands[29]. Despite these findings, there is no clear evidence for a melatonin-opioid system interaction or melatonergic ligand binding to opioid receptors. We might suggest a possible involvement of delta-opioid receptors in the antinociceptive activity of melatonin, as it was shown that its antihyperalgesic effect was partially inhibited by the selective delta opioid receptor antagonists, naltrexone and naltrindole, but not by a selective kappa opioid receptor antagonist 5'-guanidinonaltrindole[7]. Another possible explanation is that melatonin may interfere with the neural mechanisms involved in the development of tolerance to a delta-opioid agonist analgesia *via* its receptor[30]. Recently Wang reported that co-activating delta-opioid receptor and melatonin receptor could induce much longer analgesia by deltorphin-5-methoxytryptamine chimeric opioid peptides[31].

Some reports suggest that the interaction between melatonergic and opioidergic system may be explained at a molecular level. Melatonin may regulate changes in pain threshold by modulating fluctuations in opioid receptor expression and the release of beta-endorphin from pituitary gland[32-34]. It can be also suggested that melatonin, similarly to opioids, may modify K^+ and Ca^{2+} ion channel function[35,36]. Furthermore, melatonin and opioid receptors are coupled to G_i proteins and may share the Gi-mediated intracellular pathways[27,37].

Rapid elimination of melatonin from the circulation is a major obstacle in its potential use in clinical treatment. The reported half-life of melatonin in blood is approximately 20-30 min[38-40]. However, administration of higher doses of melatonin induces a nocturnal decrease in body temperature and may desensitizes its receptors[41,42]. A possible solution to overcome limited melatonin bioavailability is by developing formulations with prolonged-release or the design of synthetic melatonergic agonists with prolonged action. A controlled-release melatonin formulation (Circadin®) has recently been approved in the European Union and other countries for the treatment of insomnia in patients aged 55 years and older[43].

Neu-P11, one of the novel melatonergic agonists used in our study was shown to be a melatonin agonist, serotonin 5-HT-1A and 5-HT-1D agonist and serotonin 5-HT2B antagonist[44]. Sleep promoting, anti-diabetic, antihypertensive, analgesic, anti-neurodegenerative, anxiolytic and antidepressant effects of Neu-P11 have been demonstrated in a series of relevant animal models[15,16]. The pharmacological actions of Neu-P12, which is a close derivative of Neu-P11, have not been fully studied yet. In the present study we report that both Neu-P12 and Neu-P11 display potent antinociceptive effects and have longer duration of action compared to melatonin and might thus become potential analgesics for future use in clinical treatment of visceral pain of abdominal origin. From our experience Neu-P12 has the longest duration of action and may thus be the most promising compound fur further development. It is finally important to mention that the effects are seen following *ip* or oral application as especially the oral activity is a good prerequisite for future human use as oral application seems the most favorable delivery pathway.

Since melatonin receptors are also involved in the regulation of gastrointestinal motility, drugs targeting these receptors might be useful in the treatment of motility disorders where increased motility is a problem as in diarrhea or IBS. Thus, the effects of melatonin and its agonists on gastrointestinal motility worth additional research.

In summary melatonin, and its agonists Neu-P11, and Neu-P12 decreased visceral abdominal pain-related behaviors in mice. The antinociceptive action of the melatonergic agonists was mediated by central and peripheral opioid receptors, as well as central MT2 receptors. All analgesic effects were seen following oral application, an attribute that allows oral treatment if further developed for clinical use.

Neu-P12, which displayed the most significant analgesic effect and the longest duration of antinociceptive action and Neu-P11 have the potential to become future drugs for the treatment of pain in abdominal diseases including, but not limited to, IBS and ulcerative colitis.

COMMENTS

Background

Melatonin was reported to attenuate pain in animals and in patients with irritable bowel syndrome. However, due to low metabolic stability, it is rapidly degraded in physiological conditions.

Research frontiers

The aim was to characterize the antinociceptive action of the novel melatonin receptor agonists, Neu-P11 and Neu-P12 in animal models of visceral pain and to compare their effects to that of melatonin.

Innovations and breakthroughs

Orally and intraperitoneally administered melatonin, Neu-P11, and Neu-P12 reduced pain responses in a dose-dependent manner. Neu-P12 was more effective and displayed longer duration of action compared to melatonin. The antinociceptive effects of Neu-P11 or Neu-P12 were antagonized naloxone. Centrally, but not intraperitoneally administration of melatonin receptor antagonists, luzindole or 4-P-PDOT blocked the antinociceptive actions of Neu-P11 or Neu-P12.

Applications

Neu-P12 produced a potent and long-lasting antinociceptive effect after intraperitoneal and oral administration. Further development of this novel compound for future treatment of abdominal pain seems promising.

Terminology

Metabolic stability, resistance to degradation and attenuation of pharmacological action by various physiological factors, mainly enzymes. Irritable bowel syndrome, gastrointestinal disease characterized by motility disorders and abdominal pain.

Peer review

The authors used the percentage of control (numbers of pain-related behaviors) instead of true numbers. An interesting study and the discussion is well-done.

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BRIEF ARTICLE

Bax protein may influence the invasion of colorectal cancer

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Abstract

AIM: To evaluate the expression of Bcl-xL, Bak, and Bax proteins in correlation with particular clinico-histopathological parameters, including tumor invasion front, in patients with colorectal cancer.

METHODS: The expression of these proteins was evaluated with the use of the immunohistochemical method in 50 primary tumors.

RESULTS: According to observations, a low expression of Bax and Bak proteins is related to the localization of the tumor in the rectum ($P < 0.05$ and $P < 0.05$ respectively), which may explain an increased incidence of colorectal cancer in this area. A positive expression of Bax protein also correlates with the presence of cancer cell infiltration to lymph and blood vessels ($P < 0.05$), which may suggest the participation of this protein in the early stages of colorectal cancer progression. Moreover, a positive expression of Bcl-xL protein correlated with a positive expression of Bak protein. This may suggest a greater participation of Bcl-xL protein in the inhibition of the proapoptotic Bak protein, but not the Bax protein.

CONCLUSION: Bax protein is probably very significant in the cancerogenesis mechanism in the large intestine.

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Key words: Bak; Bax; Bcl-xL; Cancer cell infiltration; Colorectal cancer

Core tip: We determined the expressions of Bax, Bak, and Bcl-xL proteins in colorectal cancer tissue. We reported that low expression of Bax and Bak proteins is related to the localization of the tumor in the rectum, which may explain an increased incidence of colorectal cancer in this area. We also presented the participation of Bax protein in the early stages of colorectal cancer progression. Moreover, our results suggest a greater participation of Bcl-xL protein in the inhibition of the proapoptotic Bak protein, but not the Bax protein. Therefore, Bax protein is probably very significant in the carcinogenesis mechanism in the large intestine.

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INTRODUCTION

In Poland, colorectal cancer is the second most common cause of malignant cancer death in men (after bronchial

and lung cancers), as well as the third most common in women (after breast and lung cancers). Although the incidence rate in Poland is relatively low, colorectal cancer is becoming increasingly common. The worldwide approximate percentage of 5-year-survival is about 45%, whereas in Poland it is only 25%. Therefore, both prophylactics and improvement in diagnostic tests and cancer therapy are crucial.

Apoptosis is controlled by various cellular signals from within or without the cell, and thus apoptosis may have two paths (internal and external). The intracellular apoptosis path is regulated by the family of Bcl-2 proteins which control cytochrome c release from the mitochondria. The family of Bcl-2 proteins can be divided into two groups: apoptosis inducing (proapoptotic Bax, Bak, Bid, Bad, and Bok) and apoptosis impeding (antiapoptotic Bcl-2, Bcl-xL, and Bcl-w). The mechanism of apoptosis induction depends on the quantitative ratio of the proapoptotic proteins to the antiapoptotic proteins. The major impeding proteins are Bcl-2 and Bcl-xL. They connect with Bax and Bak domains, divide them, and prevent their oligomerization. This way they block the mitochondrial apoptosis path. Their overexpression strongly impedes apoptosis due to cytotoxic damage by elimination of free radicals, prevention of mitochondrial canal formation, and the release of c cytochrome[1-3].

Apoptosis plays a significant role in the prevention of cancer development in the body. As the cell cannot undergo apoptosis due to mutation or impediment by biochemical factors, it continues to divide and thus transforms into a tumor. At this point, the most important factor is *Bcl-2* oncogene, as its mutations lead to tumor development. A number of studies have shown a gradual increase in the expression of this antiapoptotic protein in the adenoma-adenocarcinoma sequence, while others have suggested that Bcl-2 expression increases only at the early stages of carcinogenesis and then decreases at the later stages. Apart from Bcl-2, the overexpression of the antiapoptotic Bcl-xL also leads to the protection of cancer cells from death. Bax protein, which is a promoter of cell death, is inactivated in cancer cells. According to studies, as much as 50% of colorectal cancer cases have the *BAX* gene mutation, which is related to the decrease in the apoptotic index in cancer cells. Moreover, the relationship of Bcl-xL and Bax proteins, which is decreased in the normal epithelium of the intestinal mucous membrane, is a crucial factor in the regulation of apoptosis. The expression of Bcl-xL in adenocarcinoma increases while Bax and Bak decrease, which is related to the cancer cells' ability to escape from death[4-6].

Therefore, the aim of this work was to evaluate the expression of Bcl-xL, Bak, and Bax proteins in correlation with particular clinico-histopathological parameters including tumor invasion front.

MATERIALS AND METHODS

Study group

The study group consisted of 50 patients with colorectal carcinoma, operated on in the Surgical Department of the J. Śniadecki Hospital in Bialystok. Colorectal carcinoma specimens and 17 control samples of normal colorectal mucosa were examined. Sections, 4 μm-thick, were cut from paraffin blocks and stained with hematoxylin and eosin (H and E). The routine histopathological assessment of the sections referred to the histological type, malignancy grade (G), clinico-pathological pTN status, regional lymph node involvement, presence of distant metastases, lymphocytic infiltration, vascular invasion, and tumor budding[7]. Inflammatory lymphocytic infiltration was graded as: 0-lacking; 1-weak, diffused; 2-moderate; 3-strong, lymphocyte nests, according to the Jass classification[8]. Lymphatic and venous invasions were examined and assessed together as vascular invasion as in the Guzińska-Ustymowicz[9] article. Tumor budding was defined as the presence of isolated single undifferentiated cancer cells, or as clusters of five or more cancer cells forming microtubular cancerous glands scattered in the stroma at the invasive margin of colorectal cancer[10]. The study protocol was approved by the local Bioethics Committee.

Immunohistochemical analysis

Formalin-fixed and paraffin-embedded tissue specimens were cut on a microtome into 4 μm sections. The sections were deparaffinized in xylenes and hydrated in alcohols. To visualize the antigen, the sections were heated in a microwave oven for 15 min in a citrate buffer (pH = 6.0). They were incubated with 3% hydrogen peroxide solution in order to block endogenous peroxidase. Next, incubation was performed with antibodies against Bcl-xL (goat polyclonal anti-human antibody, Sc-7122, Santa Cruz Biotechnology-incubation 1 h, dilution 1:300), Bak (goat polyclonal anti-human antibody, Sc-1035, Santa Cruz Biotechnology-incubation 1 h 30 min, dilution 1:150), and Bax (goat polyclonal anti-human antibody, Sc-526-G, Santa Cruz Biotechnology - incubation 1 h 30 min, dilution 1:100). The reaction was carried out using the EnVision FLEX two-step visualization system (DAKO, Poland). A color reaction for peroxidase was developed with chromogene DAB (DAKO, Poland). Bcl-xL, Bak, and Bax expression were determined by two independent pathologists using the semiquantitative method and assessed as strong (reaction visible in $\geqslant$ 25% of tumor cells) or weak (lack of reaction or the reaction present in $<$ 25% of cancer cells). Positive reactions were calculated in at least 500 cancer cells in each tissue specimen under a light microscope (× 400) (Figure 1).

Statistical analysis

Statistical analysis was conducted using Student's *t* test and the Fisher exact test. A *P* value of $<$ 0.05 was considered statistically significant. Missing data were removed in pairs.

RESULTS

Positive expression was observed as follows: Bcl-xL

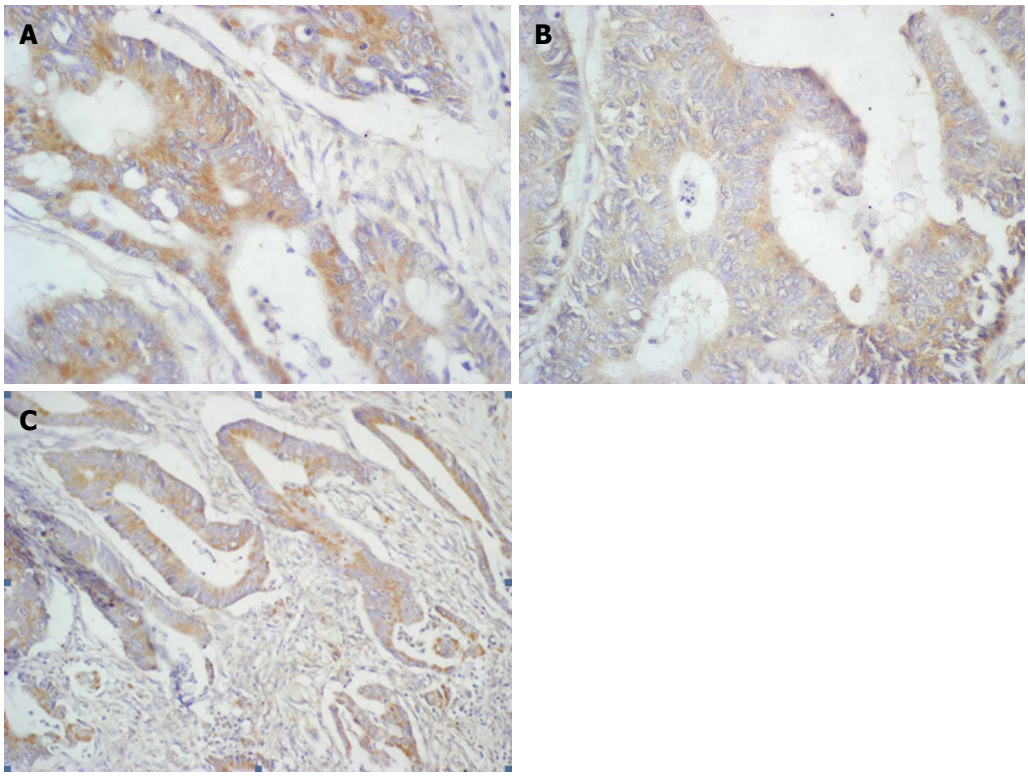

Figure 1 Positive immunohistochemical expression of Bcl-xl (A), Bak (B), and Bax (C) proteins in the main mass of colorectal carcinoma. Magnification × 400, × 400, × 100, respectively.

protein: 64% of the patients; Bax protein: 42% of the patients; and Bak protein: 40% of the patients. Statistical analysis showed no statistically significant dependencies between the expression of these proteins and the following parameters: age, gender, histological type of neoplasm, pT stage, presence of metastases to regional lymph nodes, presence of distant metastases, infiltration from inflammatory cells, and tumor budding. According to observations, low expression of Bax and Bak proteins is related to the location of the tumor in the rectum ($P < 0.05$ and $P < 0.05$ respectively). Moreover, positive Bax expression correlates with the presence of cancer cell infiltration to lymph and blood vessels ($P < 0.05$) (Tables 1 and 2). According to the statistical analysis of mutual correlations, Bak expression correlated with Bcl-xL expression ($P < 0.01$) (Table 3). In 19 patients with positive Bak expression, a positive Bcl-xL was also observed in 94.74% (18/19 patients). No similar dependency was observed in the case of Bax protein.

DISCUSSION

It has been shown that the abnormalities in the expression of proteins which regulate programmed cell death, namely proapoptotic proteins (among others Bak, Bax, and Bid) and antiapoptotic proteins (among others Bcl-2 and Bcl-xL), lead to the formation and development of cancer.

According to the literature, the expression of the proapoptotic Bak protein in a normal mucous membrane of the large intestine is observed in the cells which are localized on the crypt surface. Bak expression in adenocarcinoma of the large intestine affects a deeper area of the crypts. As regards the pattern of malignant cancer of the large intestine, Bak expression is lower compared to the normal mucous membrane and it is also dispersed irregularly. In a normal mucous membrane of the large intestine, the expression of Bax protein is absent in the crypt base and increases towards the villa. Cancer cells in a malignant primary tumor of the large intestine show medium to strong Bax expression. As regards metastases to the liver, a strong decrease in Bax expression is observed. Moreover, Bax expression is lower compared to the normal intestinal mucous membrane[11-14]. The expression of the antiapoptotic Bcl-xL protein in most cancer cells is strong, while no expression of this protein is observed in most of the glandular and well-differentiated parts of colorectal cancer. The increase in Bcl-xL protein level is also related to the more advanced tumors T3 and T4, as well as the tumors which give metastases to lymph nodes and distant organs. According to this study, Bcl-xL protein plays a significant role in apoptosis impediment, which is related to a longer survival of neoplastic cells and cancer progression[15-18].

According to scientific documentation, a positive expression of the antiapoptotic Bcl-xL protein is observed in a higher number of patients (64%) compared to the proapoptotic Bax and Bak proteins (42% and 40% patients, respectively). Bcl-xL protein can form heterodimers with Bak and Bax proteins, and thus prevent

Table 1 Relationship between proapoptotic Bax and Bak proteins and clinico-pathological parameters *n* (%)

Variables		Bax expression		*P* value	Bak expression		*P* value
		Weak	Strong		Weak	Strong	
Age	⩽ 60	5 (50)	5 (50)	0.566	5 (50)	5 (50)	0.470
	> 60	24 (60)	16 (40)		25 (62.5)	15 (37.5)	
Gender	Male	14 (60,9)	9 (39.1)	0.704	15 (65.2)	8 (34.8)	0.487
	Female	15 (55.6)	12 (44.4)		15 (55.6)	12 (44.4)	
Localization	Colon	12 (42.9)	16 (57.1)	< 0.050	13 (46.4)	15 (53.6)	< 0.050
	Rectum	17 (77.3)	5 (22.7)		17 (77.3)	5 (22.7)	
Adenocarcinoma type	Non-mucinous	28 (62.2)	17 (37.8)	0.069	27 (60)	18 (40)	1.000
	Mucinous	1 (20)	4 (80)		3 (60)	2 (40)	
pT stage	2	3 (100)	0 (0)	0.128	3 (100)	0 (0)	0.144
	3	26 (55.3)	21 (44.7)		27 (57. 5)	20 (42.5)	
Lymph node metastasis	Absent	17 (60.7)	11 (39.3)	0.660	18 (64.3)	10 (35.7)	0.485
	Present	12 (54.5)	10 (45.5)		12 (54.5)	10 (45.5)	
Distant metastasis	Absent	27 (60)	18 (40)	0.390	26 (57.8)	19 (42.2)	0.335
	Present	2 (40)	3 (60)		4 (80)	1 (20)	
Lymphocytic infiltration	Absent	2 (40)	3 (60)	0.064	4 (80)	1 (20)	0.562
	Weak	14 (56)	11 (44)		14 (56)	11 (44)	
	Moderate	13 (76.5)	4 (23.5)		11 (64.7)	6 (35.3)	
	Strong	0 (0)	3 (100)		1 (33.3)	2 (66.7)	
Vascular invasion	Absent	17 (73.9)	6 (26.1)	< 0.050	15 (65.2)	8 (34.8)	0.487
	Present	12 (44.4)	15 (55.6)		15 (55.6)	12 (44.4)	
Tumor budding	Absent	7 (63.6)	4 (36.4)	0.668	8 (72.7)	3 (27.3)	0.329
	Present	22 (56.4)	17 (43.6)		22 (56.4)	17 (43.6)	

Relationships were considered significant at $P < 0.05$. Significant relationships are marked in bold. Missing data were removed in pairs.

Table 2 Relationship between antiapoptotic protein Bcl-xL and clinico-pathological parameters *n* (%)

Variables		Bcl-xL expression		*P* value
		Weak	Strong	
Age	⩽ 60	3 (33.3)	6 (66.7)	0.853
	> 60	15 (36.6)	26 (63.4)	
Gender	Male	9 (37.5)	15 (62.5)	0.831
	Female	9 (34.6)	17 (65.4)	
Localization	Colon	8 (28.6)	20 (71.4)	0.216
	Rectum	10 (45.5)	12 (54.5)	
Adenocarcinoma type	Non-mucinous	16 (35.6)	29 (64.4)	0.844
	Mucinous	2 (40)	3 (60)	
pT stage	2	2 (66.7)	1 (33.3)	0.253
	3	16 (34)	31 (66)	
Lymph node metastasis	Absent	8 (29.6)	19 (70.4)	0.309
	Present	10 (43.5)	13 (56.5)	
Distant metastasis	Absent	14 (31.8)	30 (68.2)	0.095
	Present	4 (66.7)	2 (33.3)	
Lymphocytic infiltration	Absent	2 (40)	3 (60)	0.423
	Weak	8 (32)	17 (68)	
	Moderate	8 (47.1)	9 (52.9)	
	Strong	0 (0)	3 (100)	
Vascular invasion	Absent	10 (43.5)	13 (56.5)	0.309
	Present	8 (29.6)	19 (70.4)	
Tumor budding	Absent	5 (45.4)	6 (54.5)	0.459
	Present	13 (33.3)	26 (66.7)	

Relationships were considered significant at $P < 0.05$. Significant relationships are marked in bold. Missing data were removed in pairs.

their oligomerization and impede apoptosis. Therefore, a strong overexpression of the antiapoptotic proteins is significant in the carcinogenesis of colorectal cancer. According to this study, a positive Bcl-xL expression correlated with a positive Bak expression, however such dependency was not observed in the case of Bax protein. This may suggest a greater participation of Bcl-xL protein in the impediment of the proapoptotic Bak protein, but not Bax. This study may confirm the results obtained by other authors who have observed a decrease in Bak expression and an increase in Bax expression in neoplastic tissue[11-14]. Thus, Bax protein probably plays a very significant role in the carcinogenesis mechanism in the large intestine.

According to this study, a statistically-significant dependency between a positive expression of the proapop-

Table 3 Correlations between apoptotic proteins: antiapoptotic Bcl-xL protein and proapoptotic Bak and Bax proteins in colorectal cancer *n* (%)

	Bcl-xL expression		*P* value	Bak expression		*P* value
	Weak	Strong		Weak	Strong	
Bak weak	15 (51.72)	14 (48.28)	< 0.010	19 (65.52)	10 (34.48)	0.349
strong	1 (5.26)	18 (94.74)		11 (52.38)	10 (47.62)	
Bax weak	11 (39.29)	17 (60.71)	0.300			
strong	5 (25)	15 (75)				

Relationships were considered significant at $P < 0.05$. Significant relationships are marked in bold. Missing data were removed in pairs.

totics Bax and Bak and tumor location was observed. Particularly, a negative expression of these proteins was related with the tumor location in the rectum (77.3% and 77.3% of the patients, respectively). In cases of patients with the tumor located in other parts of the large intestine, the expression of Bax and Bak proteins was positive in 57.1% and 53.6% of patients, respectively. This may indicate a greater incidence of colorectal cancer located in the rectal area. However, no such dependencies have been reported in the literature. According to this study, no relationship between Bcl-xL expression and tumor location was observed.

Studies on the relationship between apoptosis-regulating proteins and cancer histological type showed no dependencies. However, Jansson *et al*[13] observed that a decrease in Bax expression is related to poorly differentiated cancer and the mucogenic type of colorectal cancer. A similar dependency has also been observed by other authors[11,19]. According to Zhang *et al*[20], an increase in Bcl-xL expression correlated with a poorly-differentiated cancer type, which is contrary to other apoptosis proteins. Most likely, the proteins which regulate cell death are also involved in the differentiation of cancer cells, yet this process is poorly understood and requires further studies.

Statistical analysis of this work also showed a correlation between Bax positive expression and the presence of cancer cell infiltration to lymph and blood vessels. Bax protein seems to participate in colorectal cancer cell spread, which may indicate this protein's participation in the early stages of colorectal cancer progression. However, Ogura *et al*[21] observed that a positive Bax expression is related with a smaller cancer cell infiltration to lymph vessels. The most probable cause of these contradictive results is due to the study group, or difference in the methods of immunohistochemical staining and the estimation of this protein's expression.

Our study showed no dependency between the expression of the apoptosis regulating proteins, local neoplasm advancement, and the presence of colorectal metastases to regional lymph nodes and distant organs. However, apart from the results similar to this study, such dependencies have also been reported in the literature. The increase in Bcl-xL expression is associated with the presence of metastases to regional lymph nodes and the C + D stage of colorectal cancer in Duke's classification. Thus, it is suggested that Bcl-xL protein plays a significant role in the development of colorectal cancer and its metastases[20]. A decrease in the expression of Bax proapoptotic protein was related mainly with an increase in cancer stage by Duke's classification. Moreover, the percentage of positive cells in the metastasis to the liver decreased with the occurrence of primary tumor metastases to regional lymph nodes, as well as with an increase in its size[11,19]. A decreased Bax expression is related with the loss of mitochondrial apoptosis activation. This mechanism allows for easy cancer cell development. In this case, a decrease in Bax expression is connected with a poor prognosis for the patient. Moreover, scientists noticed that Bax-negative patients with colorectal cancer show a shorter survival compared to Bax-positive patients[21]. According to other scientists, a positive expression of this protein is related to a poor prognosis in patients with colorectal metastases. The discrepancies resulted mainly from different study groups. As has been mentioned, these proteins play a more or less significant role depending on the colorectal cancer stage. Therefore, changes in their expression give different prognoses in different study groups.

No dependencies between the expression of Bcl-xL, Bax, and Bak and the presence of buds in the tumor invasion front were observed. The presence of tumor buds, single or small groups of invasive cancer cells in the tumor front which infiltrate the desmoplastic stroma, is an indicator of malignancy potential, including metastatic abilities. Despite reports suggesting the existence of a relationship between the expression of the antiapoptotic Bcl-2 protein and tumor budding presence, Bcl-xL, Bax, and Bak proteins seem not to be involved in the process of their formation[22].

COMMENTS

Background

Colorectal cancer is still the most common cancer in terms of incidence and cancer motility. Processes responsible for the carcinogenesis of cancers are still investigated. One of these processes is apoptosis, essential for the removal of abnormal and damaged cells. Therefore the authors wanted to present the development of resistance to apoptosis in colorectal cancer cells in correlation with clinicopathological parameters.

Research frontiers

It is known that the ratio of proapoptotic to antiapoptotic proteins in cancer cells is disrupted. The main causes of this disorder are mutations in the *Bcl-2* and *Bax* genes. However, it is unknown whether any of these proteins play a more important role in the carcinogenesis of colorectal cancer.

Innovations and breakthroughs

The authors showed that low expression of Bax and Bak proteins is related with the localization of the tumor in the rectum, which may explain an increased incidence of colorectal cancer in this area. They also presented the participation of Bax protein in colorectal cancer progression by vascular invasion. This is also the first study which presents a greater participation of the Bcl-xL protein in the inhibition of the proapoptotic Bak protein, but not the Bax protein. Therefore Bax protein is probably very significant in the carcinogenesis mechanism in the large intestine.

Applications

By demonstrating Bax proteins as an important protein involved in the regulation of apoptosis in colorectal cancer cells, this fact can be used by experts as a target in cancer therapy.

Peer review

The authors evaluated the expression of Bcl-xL, Bak, and Bax proteins in correlation with particular clinico-histopathological parameters, including tumor invasion front, in patients with colorectal cancer, and demonstrated that a low expression of Bax and Bak proteins is related with the localization of the tumor in the rectum. They also demonstrated that a positive expression of Bax protein correlates with the presence of cancer cell infiltration to lymph and blood vessels, which may suggest the participation of this protein in the early stages of colorectal cancer progression. This manuscript is very interesting and provides novel evidence.

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BRIEF ARTICLE

Reduced hemoglobin and increased C-reactive protein are associated with upper gastrointestinal bleeding

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Abstract

AIM: To investigate the early upper gastrointestinal endoscopy (endoscopy) significantly reduces mortality resulting from upper gastrointestinal (GI) bleeding.

METHODS: Upper GI bleeding was defined as 1a, 1b, 2a, and 2b according to the Forrest classification. The hemoglobin (Hb), and C-reactive protein (CRP) were examined at around the day of endoscopy and 3 mo prior to endoscopy. The rate of change was calculated as follows: (the result of blood examination on the day of endoscopy - the results of blood examination 3 mo prior to endoscopy)/(results of blood examination 3 mo prior to endoscopy). Receiver operating characteristic curves were created to determine threshold values.

RESULTS: Seventy-nine men and 77 women were enrolled. There were 17 patients with upper GI bleeding: 12 with a gastric ulcer, 3 with a duodenal ulcer, 1 with an acute gastric mucosal lesion, and 1 with gastric cancer. The area under the curve (AUC), threshold, sensitivity, and specificity of Hb around the day of endoscopy were 0.902, 11.7 g/dL, 94.1%, and 77.1%, respectively, while those of CRP were 0.722, 0.5 mg/dL, 70.5%, and 73%, respectively. The AUC, threshold, sensitivity, and specificity of the rate of change of Hb were 0.851, -21.3%, 76.4%, and 82.6%, respectively, while those of CRP were 0.901, 100%, 100%, and 82.5%, respectively.

CONCLUSION: Predictors for upper GI bleeding were Hb < 11.7 g/dL, reduction rate in the Hb > 21.3% and an increase in the CRP > 100%, 3 mo before endoscopy.

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Key words: Receiver operating characteristic; Area under curve; Sensitivity; Specificity; Gastrointestinal bleeding

Core tip: Mortality rate of upper gastrointestinal (GI) bleeding is approximately 10%. Early upper gastrointestinal endoscopy significantly reduces mortality. We attempted to search for predictors of bleeding. Patient records were analyzed retrospectively. The rate of change was calculated as follows: (the result of blood examination on the day of endoscopy - the results of blood examination 3 mo prior to endoscopy)/(results of blood examination 3 mo prior to endoscopy). Receiver

operating characteristic curves were created to determine threshold values. Predictors for upper GI bleeding were hemoglobin (Hb) < 11.7 g/dL, reduction rate in the Hb > 21.3% and an increase in the C-reactive protein > 100%, 3 mo before endoscopy.

Tomizawa M, Shinozaki F, Hasegawa R, Togawa A, Shirai Y, Ichiki N, Motoyoshi Y, Sugiyama T, Yamamoto S, Sueishi M. Reduced hemoglobin and increased C-reactive protein are associated with upper gastrointestinal bleeding. *World J Gastroenterol* 2014; 20(5): 1311-1317 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1311.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1311

INTRODUCTION

Upper gastrointestinal (GI) tract bleeding is diagnosed and treated with upper gastrointestinal endoscopy (endoscopy)[1]. Longstreth reported an annual incidence rate of 102.0 acute upper GI bleeding hospitalizations per 100000 population from a San Diego health maintenance organization; 61.6% of the patients were diagnosed with peptic ulcers[2]. Patients with bleeding are treated endoscopically using endoscopic clipping and bipolar electrocoagulation[3]. Endoscopic clipping significantly reduces recurrent bleeding as compared with epinephrine[4]. For patients who do not respond to these therapies, arteriographic embolization is performed[5,6]. Transcatheter embolization is a second line for patients failure of endoscopical hemostasis particularly in poor condition[7]. Despite these advances, mortality from the disorder has remained at approximately 10%[8]. The disease often occurs in patients using antiplatelet agents and anticoagulants.

Early endoscopy significantly reduces the mortality by peptic ulcer bleeding[9]. Patients at risk factors for mortality should be assessed for early endoscopy[10]. Clinical predictors of increased risk for mortality are age > 65 years, shock, poor overall health status, low initial hemoglobin (Hb) levels, melena, transfusion requirement, fresh red blood on rectal examination and nasogastric aspirate, sepsis, and elevated blood urea nitrogen (BUN) or creatinine (Cr) levels[11]. However, there are no data regarding the threshold levels of Hb, BUN, and Cr to assess patients for the risk of bleeding prior to endoscopy.

Mahlknecht *et al*[12] report age-related change of Hb. They excluded patients who had hematological history, oncological diseases, chronic infection or inflammation. They show that Hb decreases as aging, and that average Hb is, interestingly, below normal range of Hb. When patients with low Hb are encountered, Hb of previous days is always checked. Decreasae in Hb suggested that the patients had bleeding diseases, such as gastric ulcers. Therefore, we analyzed the change rate of Hb and other parameters.

Therefore, we attempted to evaluate the usefulness of Hb, BUN, and Cr levels as predictors of bleeding. The white blood cell (WBC) count and C-reactive protein (CRP) levels were also evaluated because they were useful indicators of inflammation including peptic ulcers[13].

MATERIALS AND METHODS

In our hospital, standard Olympus endoscopes (GIF Q260, GIF XQ260) (Olympus, Tokyo, Japan) were used. Patients with bleeding were subjected to endoscopic clipping (HX-610-135) (Olympus) or argon plasma coagulation (APC300) (ERBE, Tokyp, Japan).

Patient records were analyzed retrospectively from April 2012 to March 2013. During this period, 1101 patients underwent upper GI endoscopy (endoscopy). Indications of 1101 endoscopies were screening, anemia, examination of abdominal symptoms and other reasons. We chose patients who had laboratory data around the day of endoscopy and 3 mo before endoscopy to analyze reduction rate. Patient number, thus, reduced to 156 patients. Seventeen patients with bleeding matched the criteria and were analyzed. The other patients were excluded from the present study. No patients were thalassemia or sickle cell anemia. Bleeding was found in 15 patients with a peptic ulcer, 1 patient with an acute gastric mucosal lesion (AGML), and 1 patient with gastric cancer. Bleeding was classified according to the Forrest classification[14]. Patients were categorized into the bleeding group when they met the Forrest classification because our goal was the assessment of upper GI bleeding; therefore, subjects were not restricted to those with peptic ulcers. In brief, active bleeding lesions include spurting vessels (1a), oozing vessels (1b), visible vessels (2a), and clots (2b). Patients diagnosed as 1a, 1b, 2a, and 2b were classified into the bleeding group (15 patients), and all others made up the non-bleeding group (142 patients). Gastritis was diagnosed according to the Sydney system[15]. Blood examination results were analyzed between 2 d before endoscopy and the day of endoscopy (defined as around the day of endoscopy). Data were also collected 3 mo before endoscopy to analyze the rate of change in the results. Patients were enrolled only when their data were available, around the day of endoscopy and 3 mo prior to endoscopy. The parameters analyzed in the blood examination were WBC ($\times 10^4$ cells/μL), Hb (mg/dL), CRP (mg/dL), Cr (mg/dL), and BUN (mg/dL) levels. Our study was subjected to our institutional ethics committee, and approved. It was not considered a clinical trial because it was performed as part of routine clinical practice. Patient anonymity was preserved.

The rate of change was calculated as follows: (the results of blood examination on the day of endoscopy-the results of blood examination 3 mo prior to endoscopy)/(results of blood examination 3 mo prior to endoscopy). Receiver operating characteristic (ROC) curves were created with JMP 8.0.2 (SAS Institute Japan, Tokyo, Japan). We investigated the Hb, CRP, Cr, and BUN levels, and WBC count around the day of endoscopy. The rate of change in the WBC count, and Hb, CRP, Cr, and BUN levels was also analyzed. The area under the curve (AUC)

Table 1 Endoscopic diagnosis and patient number

Diagnosis	Number of patients	Number of patients with bleeding	Forrest classification			
			1a	1b	2a	2b
Gastric ulcer	38	12	1	1	5	5
Duodenal ulcer	9	3	0	1	2	0
AGML	1	1	1	0	0	0
Gastric cancer	5	1	0	0	1	0
Esophageal varix	1	0	NA	NA	NA	NA
Gastritis	50	0	NA	NA	NA	NA
Esophageal hiatus hernia	2	0	NA	NA	NA	NA
Gastric polyp	5	0	NA	NA	NA	NA
Submucosal tumor	1	0	NA	NA	NA	NA
Normal	45	0	NA	NA	NA	NA
Total	157	17	2	2	8	5

AGML: Acute gastric mucosal lesion; NA: Not applicable.

was used as a measure of diagnostic efficacy. The threshold value was determined as the highest sensitivity and specificity and calculated automatically by the software on the location where a line with a slope of 45° contacted the ROC curve. The Wilcoxon signed rank test was used for statistical analysis, and $P < 0.05$ was considered statistically significant. Adobe Illustrator CS4 (Adobe Systems, San Jose, CA) was used to make figures.

RESULTS

Seventy-nine patients were women (age, 68.2 ± 11.5 years, mean ± SD), and 77 were men (age, 70.5 ± 12.6 years). Table 1 presents the patient number, endoscopic diagnosis, and Forrest classification. Bleeding was noted in 17 patients: 12 patients with a gastric ulcer, 3 patients with a duodenal ulcer, 1 patient with an AGML, and 1 patient with gastric cancer. Spurting-type bleeding (1a) was found in 1 patient with a peptic ulcer and 1 patient with an AGML. Their bleeding was controlled using endoscopic clipping. The other patients with bleeding were treated with endoscopic clipping or argon plasma coagulation. None of the patients died in our study. Table 2 shows laboratory data of each disease. Table 3 shows the comparison of each parameter between the bleeding and non-bleeding groups. WBC, BUN and Cr levels, and BUN/Cr were higher in the bleeding group than in the non-bleeding group, but the difference was not significant. Hb ($P < 0.0001$) level was lower and CRP ($P = 0.009$) level was higher in the bleeding group than in the non-bleeding group, with a statistical significance difference. Hb and CRP were candidates for threshold values to diagnose bleeding before endoscopy.

Figure 1 depicts the ROC curves of each parameter around the day of endoscopy. WBC (Figure 1A), CRP (Figure 1C), BUN (Figure 1D), Cr (Figure 1E), and BUN/Cr (Figure 1F) were closer to the reference line than Hb (Figure 1B). The AUC, threshold, sensitivity, and specificity are presented in Table 4. The AUC of Hb was > 0.9, whereas that of the other parameters was lower < 0.8. The threshold value of Hb was 11.7 g/dL. For this value, sensitivity and specificity were 94.1% and 77.1%, respectively. The AUC of CRP was 0.722, which was lower than expected. The threshold value of CRP was 0.5 mg/dL. Sensitivity and specificity of this value were 70.5% and 73%, respectively. Sensitivity and specificity did not seem useful for predicting bleeding before endoscopy.

Figure 2 depicts the ROC curve of the rate of change of each parameter between around the day of endoscopy and 3 mo prior to endoscopy. WBC (Figure 2A) and Cr (Figure 2E) were far from the reference line. BUN crossed the reference line (Figure 2D). Hb (Figure 2B) and CRP (Figure 2C) were close to the reference line. The AUC, threshold, sensitivity, and specificity are presented in Table 5. The AUC of Hb and CRP was 0.851 and 0.901, respectively. They were approximately 0.9. The other threshold values were < 0.6. The threshold of Hb was -21.3%. For this value, sensitivity and specificity were 76.4% and 82.6%, respectively. The threshold value of CRP was 100%. Sensitivity and specificity for this value were 100% and 82.5%, respectively.

DISCUSSION

A low Hb level is strongly associated with upper GI bleeding. Wong *et al*[16] followed up with 3386 patients with bleeding peptic ulcers after initial hemostasis. They found that an Hb level of < 10 g/dL is a predictive value for rebleeding (OR = 1.87; 95%CI: 1.18-2.96). Heldwein *et al*[17] reported that Forrest 1a and 2a classifications are associated with a poor prognosis, and a decreased Hb level and a requirement for blood transfusion are prospective values for emergency endoscopy. These reports focus on the prediction of rebleeding or emergency. In our study, the correlation between blood examination and bleeding diagnosed with endoscopy was analyzed. Our data clearly show that a low Hb level indicated bleeding from the upper GI tract. Similar to our study design, Giese *et al*[18] retrospectively analyzed the medical records of patients who underwent emergency endoscopy. The relative risks (*P* value) of the WBC count (> 12000/μL) and Hb (< 8.0 g/dL) level for bleeding were 1.18 ($P = 0.6$) and 1.38 ($P = 0.3$), respectively. A low Hb level did not correlate with bleeding. They excluded patients with symptoms of bleeding because their aim was to examine predictive values for bleeding. The reason of the discrepancy between the previous report and our results might be that we included patients with symptoms of bleeding such as low blood pressure and tachycardia. Srygley *et al*[19] reported that the likely ratio of Hb < 8.0 g/dL is 4.5-6.2 for severe bleeding. Our threshold value of Hb was 11.7 g/dL. The reason for this difference might be the inclusion of patients with mild bleeding. It might be proposed that Hb < 11.7 g/dL is the threshold for patients with mild and severe bleeding, and Hb < 8.0 g/dL is the threshold for those with severe bleeding.

It is hard to discriminate upper GI bleeding from lower GI bleeding with the parameters we presented. Endoscopy and colonoscopy should be performed for patients

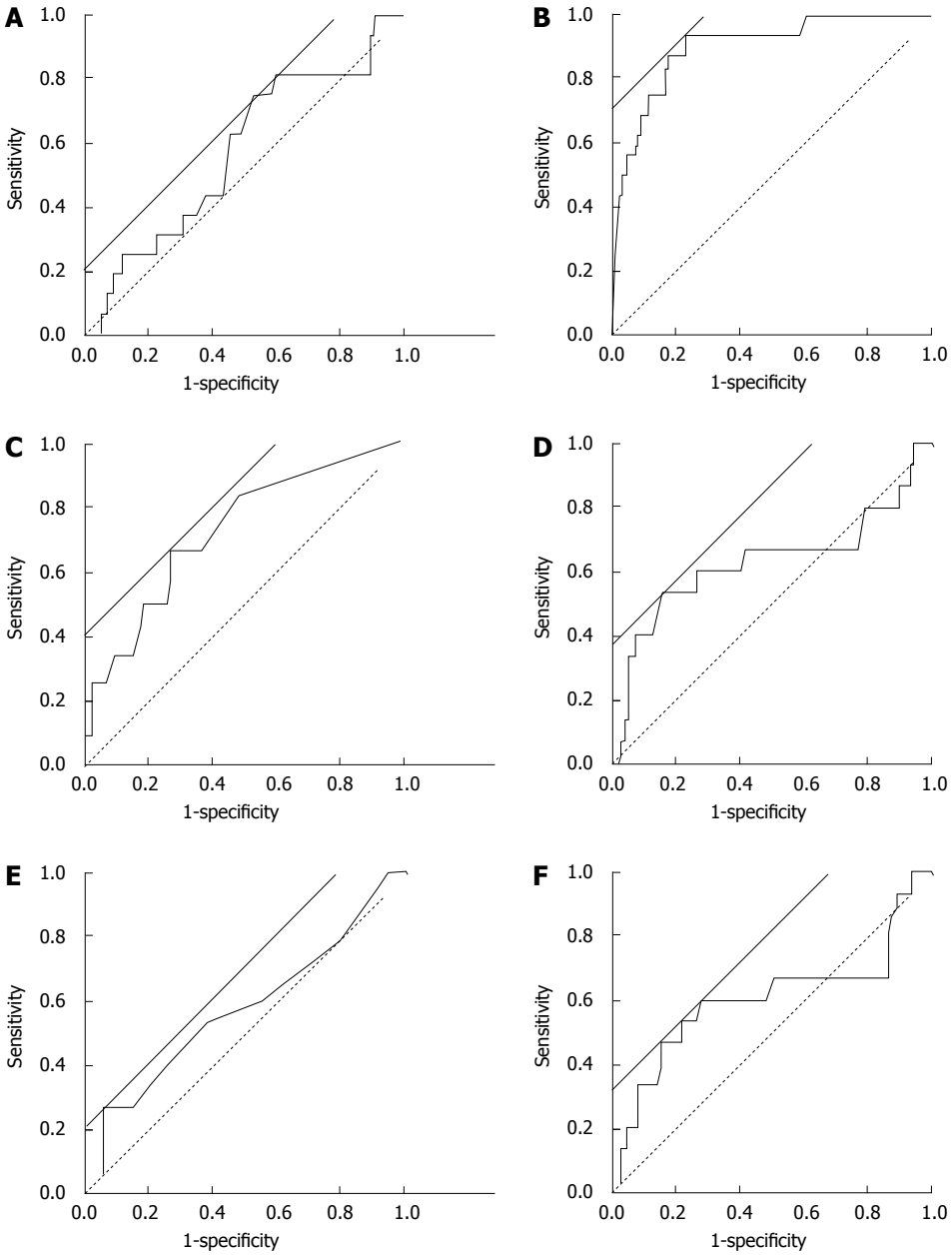

Figure 1 Receiver operating characteristic curves of the results of blood examination around the date of endoscopy. Receiver operating characteristic curves were created based on the results of blood examination around the day of endoscopy. The analyzed data were white blood cell count (A), hemoglobin (B), C-reactive protein (C), blood urea nitrogen (D), and creatinine (E), and blood urea nitrogen/creatinine (F). Solid straight line, a line with a slope of 45° to calculate threshold by the software (JMP 8.0.2); broken line, reference line.

with low Hb[20]. Low Hb suggests patients with upper or lower GI bleeding, hematological disorders, such as thalassemia and sickle cell anemia. Those patients should be investigated following Goddard *et al*[21]. Endoscopy and colonoscopy were desirable for patients suspected with upper or lower GI bleeding. Parameters analyzed in our present study suggested bleeding from upper or lower GI. It was expected to be applied to lower GI bleeding.

Erosive esophagitis, gastritis, and duodenitis are associated with a low Hb level because of slow and long-term bleeding[22]. The rate of reduction in the Hb level might be another predictor of bleeding from the upper GI tract. Pundzius[23] analyzed 659 patients whose bleeding peptic ulcer was treated endoscopically. They determined that the rate of reduction in the Hb level is a predictive value for rebleeding. In our study, the rate of reduction in the Hb level during 3 mo strongly correlated with bleeding from the upper GI tract. The previous report and our data suggested that a higher rate of reduction in the Hb level predicted bleeding prior to endoscopy. Our results clearly showed that 21.3% was the threshold of the rate of reduction rate in the Hb level.

CRP is a plasma protein produced in the liver in response to tissue inflammation[24]. Boehme *et al*[25] reported that an elevated CRP level was a positive predictive value for peptic ulcers caused by active inflammation. In the present study, CRP around the day endoscopy was performed was significantly higher in the bleeding group than

Table 2 Laboratory data of each disease

Diagnosis	WBC count (/μL)	Hb (g/dL)	CRP (mg/dL)	BUN (mg/dL)	Cr (mg/dL)	BUN/Cr
Gastric ulcer	8080 ± 4104	10.4 ± 3.2	1.17 ± 1.43	23.3 ± 14.1	1.01 ± 0.44	31.7 ± 38.4
Duodenal ulcer	5520 ± 729	10.8 ± 2.7	0.28 ± 0.21	11.0 ± 5.5	0.80 ± 0.14	14.9 ± 10.2
AGML	6600	4.8	0.1	20.7	0.9	23
Gastric cancer	5860 ± 1132	11.1 ± 2.3	0.17 ± 0.11	16.1 ± 3.2	0.80 ± 0.16	20.5 ± 4.7
Esophageal varix	4700	10.1	0.13	21.7	0.8	27.1
Gastritis	6073 ± 2032	13.4 ±1.9	0.23 ± 0.22	14.5 ±5.4	0.81 ± 0.18	18.7 ± 6.4
Esophageal hiatus hernia	8350 ± 495	11.8 ± 1.1	1.45 ± 1.77	23.5 ± 4.9	1.30 ± 0.71	20.0 ± 7.07
Gastric polyp	6480 ± 1136	13.3 ± 0.5	0.95 ± 1.20	13.4 ± 4.6	0.78 ± 0.08	17.0 ± 4.6
Submucosal tumor	5000	12	1	20	0.6	33.3
Normal	5865 ± 1516	13.3 ± 1.6	0.64 ± 0.81	14.4 ± 2.1	0.77 ± 0.19	18.4 ± 4.3

WBC: White blood cell; Hb: Hemoglobin; CRP: C-reactive protein; BUN: Blood urea nitrogen; Cr: Creatinine; AGML: Acute gastric mucosal lesion.

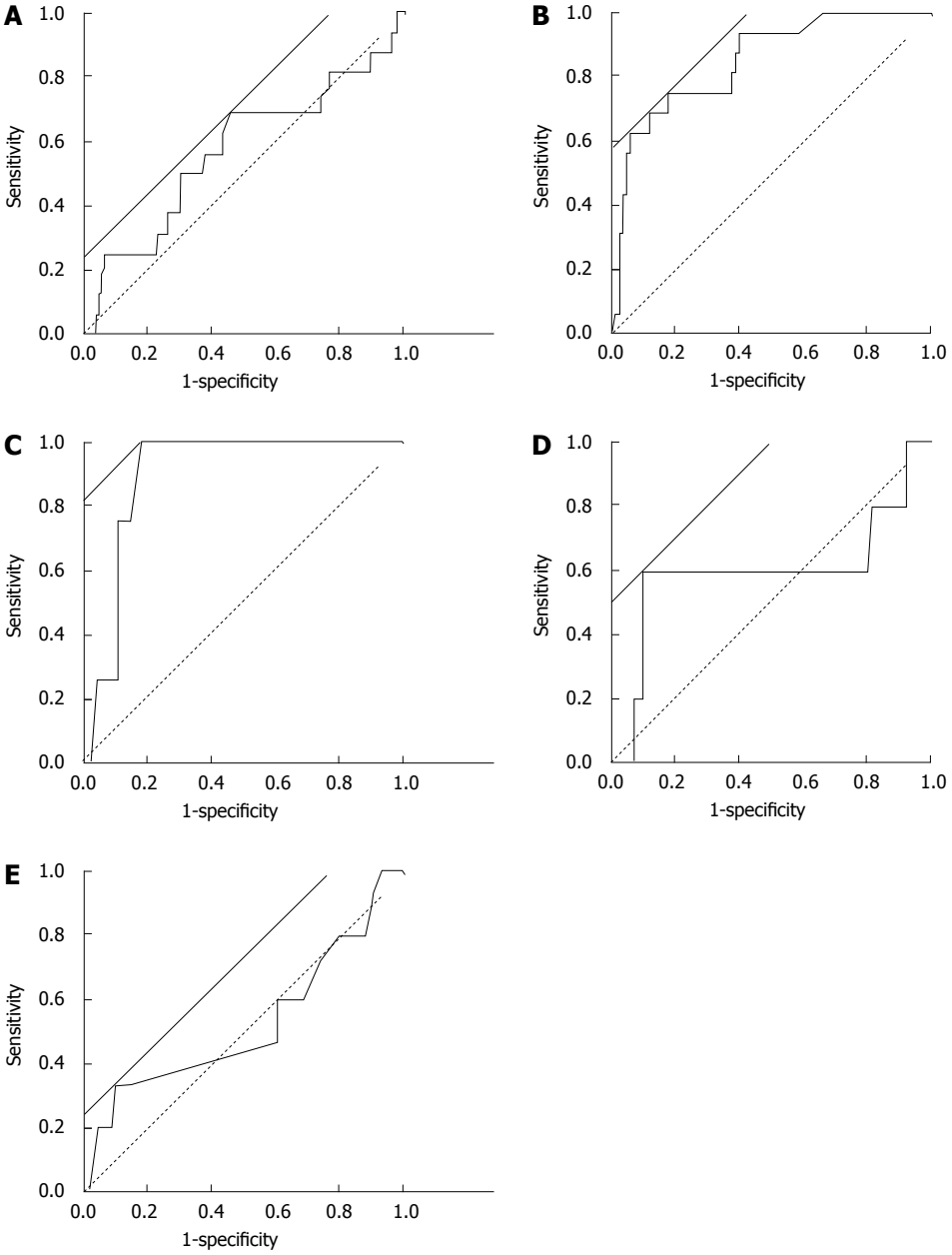

Figure 2 Receiver operating characteristic curves of the results of change rate of blood examination. Receiver operating characteristic curves were created based on the rate of change in the results of blood examination. The rate of change rate was calculated as a comparison between around the day of endoscopy and 3 mo prior to the date of endoscopy (see text). The analyzed data were white blood cell count (A), hemoglobin (B), C-reactive protein (C), blood urea nitrogen (D), and creatinine (E). Solid straight line, a line with a slope of 45° to calculate threshold by the software (JMP 8.0.2); broken line, reference line.

Table 3 Comparison between bleeding (+) and (-) around the day of endoscopy

Laboratory data	Bleeding (-)		Bleeding (+)		Wilcoxon (*P* value)
	Mean	95%CI	Mean	95%CI	
WBC count (/μL)	6370	5946-6795	6700	5613-7786	0.341
Hb (g/dL)	12.8	12.5-13.3	8.4	6.9-9.8	< 0.0001
CRP (mg/dL)	0.50	0.346-0.656	1.37	0.29-2.44	0.009
BUN (mg/dL)	16.4	14.6-18.1	22.5	15.4-29.7	0.096
Cr (mg/dL)	0.83	0.78-0.88	0.92	0.74-1.10	0.302
BUN/Cr	22.0	17.6-26.3	25.1	17.4-32.8	0.196

Bleeding (-), patients not diagnosed as 1a, 1b, 2a, or 2b by Forrest classification; bleeding (+), patients diagnosed as 1a, 1b, 2a, and 2b.Wilcoxon: Wilcoxon signed rank test; CI: Confidence interval; WBC: White blood cell; Hb: Hemoglobin; CRP: C-reactive protein; BUN: Blood urea nitrogen; Cr: Creatinine.

Table 4 Area under curve and threshold around the day of endoscopy

Laboratory data	AUC	Threshold	Sensitivity	Specificity
WBC count	0.573	5600 (/μL)	70.5%	46.4%
Hb	0.902	11.7 (g/dL)	94.1%	77.1%
CRP	0.722	0.5 (mg/dL)	70.5%	73.0%
BUN	0.635	20.7 (mg/dL)	52.9%	85.0%
Cr	0.580	1.20 (mg/dL)	35.3%	94.9%
BUN/Cr	0.605	23.0	58.8%	29.9%

AUC: Area under curve; WBC: White blood cell; Hb: Hemoglobin; CRP: C-reactive protein; BUN: Blood urea nitrogen; Cr: Creatinine; BUN/Cr: The ratio of BUN divided by Cr.

in the non-bleeding group, although the AUC was not high. The increase in the CRP level significantly correlated with bleeding. Our data indicated that a > 2-fold increase in the CRP level was indicative of bleeding. Serum CRP level correlates with serum interleukin (IL)-6 level, a key cytokine involved in the acute-phase response[26]. Tomita *et al*[27] compared the expression levels of IL-6 in the biopsy specimens of peptic ulcers with those from the antrum. They found that IL-6 is expressed more in peptic ulcers than the antrum. These reports indicate that peptic ulcer upregulates IL-6 and, consequently, increases the production of CRP. The problem with CRP is that its increase is nonspecific to peptic ulcers or upper GI bleeding. It is also used as a biomarker for predicting cardiovascular diseases[28]. The nonspecific nature of CRP might be the reason for the relatively low specificity of its rate of change, although its sensitivity was 100%. CRP, Hb, clinical symptoms, and patient conditions are cumulatively essential to predict bleeding from the upper GI tract prior to endoscopy.

A BUN/Cr ratio > 30 is a useful value to diagnose upper GI bleeding[19]. We hypothesized that BUN levels and BUN/Cr were higher in bleeding patients than in non-bleeding patients. However, according to our results, BUN and BUN/Cr did not correlate with bleeding. The reason for the discrepancy between Srygley *et al*[19] and our results is unclear. Chalasani *et al*[29] compared the BUN/Cr ratio between upper GI bleeding patients and lower GI bleeding patients. They concluded that the ratio is not useful to differentiate between the 2 types of bleeding.

Table 5 Area under curve and threshold of the rate of change rate

Laboratory data	AUC	Threshold	Sensitivity	Specificity
WBC count	0.577	6.0%	64.7%	55.3%
Hb	0.851	-21.3%	76.4%	82.6%
CRP	0.901	100%	100%	82.5%
BUN	0.592	50.3%	58.8%	89.6%
Cr	0.527	-14.3%	35.3%	90.4%

AUC: Area under curve; WBC: White blood cell; Hb: Hemoglobin; CRP: C-reactive protein; BUN: Blood urea nitrogen; Cr: Creatinine.

In conclusion, an Hb level < 11.7 g/dL indicated bleeding from the upper GI tract. Other predictors for bleeding were a > 21.3% rate of reduction in the Hb level and an increase in the CRP level by > 100%, 3-6 mo before endoscopy.

COMMENTS

Background

Upper gastrointestinal (GI) bleeding is fatal when patients are not diagnosed correctly and treated adequately. Early upper GI endoscopy (endoscopy) significantly reduces mortality. Clues for decision making of endoscopy is awaited.

Research frontiers

Clinical predictors of increased risk for mortality are age > 65 years, shock, poor overall health status, low initial hemoglobin (Hb) levels, melena, transfusion requirement, fresh red blood on rectal examination and nasogastric aspirate, sepsis, and elevated blood urea nitrogen or creatinine levels.

Innovations and breakthroughs

Threshold values of hemoglobin and C-reactive protein (CRP) to diagnose bleeding peptic ulcer were investigated with receiver operating characteristic (ROC) curves. An Hb level < 11.7 g/dL indicated bleeding from the upper GI tract. Other predictors for bleeding were a > 21.3% rate of reduction in the Hb level and an increase in the CRP level by > 100%, 3-6 mo before endoscopy.

Applications

Endoscopy is strongly recommended for patients with Hb and CRP above threshold values.

Terminology

Receiver operating characteristic: ROC is a graph that plots the fraction of true positives out of the positives true positive rate) *vs* the fraction of false positives out of the negatives false positive rate), at various thresholds.

Peer review

This study attempted to establish biomarkers for upper gastrointestinal bleeding by measuring Hb and CRP. The study design is rational. It is hard to discriminate upper and lower gastrointestinal bleeding with lower Hb and higher CRP because blood loss from any part of the body will lead to low Hb and CRP is a non-specific indicator of inflammatory response. Endoscopy is strongly recommended for patients with lower Hb and higher CRP. If upper GI does not show any evidence of bleeding, the patients should be subjected to colonoscopy. The study is of particular interest to the practical medicine.

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BRIEF ARTICLE

Risk factors for proximal migration of biliary tube stents

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Abstract

AIM: To analyze the risk factors for biliary stent migration in patients with benign and malignant strictures.

METHODS: Endoscopic stent placement was performed in 396 patients with bile duct stenosis, at our institution, between June 2003 and March 2009. The indications for bile duct stent implantation included common bile duct stone in 190 patients, malignant lesions in 112, chronic pancreatitis in 62, autoimmune pancreatitis in 14, trauma in eight, surgical complications in six, and primary sclerosing cholangitis (PSC) in four. We retrospectively examined the frequency of stent migration, and analyzed the patient factors (disease, whether endoscopic sphincterotomy was performed, location of bile duct stenosis and diameter of the bile duct) and stent characteristics (duration of stent placement, stent type, diameter and length). Moreover, we investigated retrieval methods for migrated stents and their associated success rates.

RESULTS: The frequency of tube stent migration in the total patient population was 3.5%. The cases in which tube stent migration occurred included those with common bile duct stones (3/190; 1.6%), malignant lesions (2/112; 1.8%), chronic pancreatitis (4/62; 6.5%), autoimmune pancreatitis (2/14; 14.3%), trauma (1/8; 12.5%), surgical complications (2/6; 33.3%), and PSC (0/4; 0%). The potential risk factors for migration included bile duct stenosis secondary to benign disease such as chronic pancreatitis and autoimmune pancreatitis ($P = 0.030$); stenosis of the lower bile duct ($P = 0.031$); bile duct diameter > 10 mm ($P = 0.023$); duration of stent placement > 1 mo ($P = 0.007$); use of straight-type stents ($P < 0.001$); and 10-Fr sized stents ($P < 0.001$). Retrieval of the migrated stents was successful in all cases. The grasping technique, using a basket or snare, was effective for pig-tailed or thin and straight stents, whereas the guidewire cannulation technique was effective for thick and straight stents.

CONCLUSION: Migration of tube stents within the bile duct is rare but possible, and it is important to determine the risk factors involved in stent migration.

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Key words: Migration; Endoscopic biliary stent; Risk factor; Endoscopic retrograde cholangiopancreatography; Retrieval

Core tip: Endoscopic biliary stenting with a tube stent is an accepted therapy for biliary obstruction due to malignant or benign disease. However, stent migration occurs in 5%-10% of patients undergoing biliary stenting. Therefore, it is important to know the factors affecting biliary tube stent migration. We retrospectively examined endoscopic stent placement in 396 patients with bile duct stenosis, and analyzed the frequency of stent migration and the risk factors (patient factors disease, endoscopic sphincterotomy, location of stenosis, diameter of bile duct) and stent factors (duration of place-

ment, type, diameter, length).

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INTRODUCTION

Endoscopic biliary stenting using a tube stent is an accepted therapy for biliary obstruction developing secondary to malignant or benign disease[1]. The complication rate associated with biliary stent use reportedly ranges from 8% to 10%[2-4], and the described complications include cholangitis, cholecystitis, duodenal perforation, bleeding, pancreatitis, stent fracture, proximal stent migration, distal stent dislocation, and stent occlusion resulting in recurrent biliary obstruction[5-16]. Stent migration is a rare complication associated with biliary stenting[11,17,18]. Most tube stents are pig-tailed shaped with flaps at each end, to prevent proximal or distal migration. However, stents may migrate proximally or distally as a late complication of endoscopic stenting in 5%-10% of patients who have undergone biliary stenting[19]. Proximal stent migration can cause biliary obstruction and cholangitis, thus requiring retrieval or re-stenting. Therefore, it is important to determine the factors influencing biliary tube stent migration that have not yet been documented. A few studies have reported on the risks of stent migration[17]. Johanson *et al*[17] reported that stent migration occurs in cases of cholangiocarcinoma, larger diameter stents, and short stents. In addition, they concluded that the reason for migration was malignant stricture of the bile duct or, in cases of benign disease, advancement of a stricture that was previously present. In addition, the presence of multiple stents, a broken stent, and differences in physical constitution are proposed as factors causing stent migration. The retrieval of migrated stents is technically challenging, but may usually be achieved endoscopically, by using forceps, snare, or balloon techniques, and rarely requires surgical intervention[18,20-22]. In the present study, we aimed to determine the frequency of biliary stent migration; analyze the risk factors for proximal stent migration; and describe the methods used for retrieval of migrated stents.

MATERIALS AND METHODS

Patients

Between June 2003 and March 2009, endoscopic stent placement was performed in 396 patients with bile duct stenosis at our institution. The diseases requiring bile duct stenting included common bile duct stones in 190 patients, malignant lesions in 112, chronic pancreatitis in 62, autoimmune pancreatitis in 14, trauma in eight, surgical complications in six, and primary sclerosing cholangitis (PSC) in four (Table 1).

Endoscopic therapy

Endoscopic retrograde cholangiopancreatography was performed using a JF-240, JF-260V, or TJF-260V unit (Olympus Medical Systems, Tokyo, Japan) with the patient under conscious sedation with diazepam and pethidine. We attempted to place a guidewire (Jagwire High Performance Guidewire; Boston Scientific, Natick MA, United States) across the stenotic lesion. After the guidewire was successfully placed across the stenotic lesion, intraductal ultrasonography, brushing cytology, bile juice cytology, and biopsy were performed to diagnose the disease on a case-by-case basis.

Dilation of stenosis

In the case of severe stenosis, the stenotic lesions were dilated with a dilation catheter (6-, 7-, or 9-Fr, Soehendra Biliary Dilation Catheter; Cook Medical, Bloomington, IN, United States) or a dilation balloon catheter (diameter: 6 mm, length: 2 cm; Hurricane TM RX Balloon Dilation Catheter; Boston Scientific).

Bile duct stenting

After dilation, a pig-tail shaped (Cook Medical) or a straight stent (Double layer stent; Olympus Medical Systems) was implanted in the stenotic lesion; the stents included 7- or 10-Fr polyethylene stents with multiple side holes. A bile duct stent was implanted to drain the bile juice and dilate the stenotic lesions of the bile duct. In cases involving common bile duct stones, a temporary stent was implanted in cases with residual stones, and residual stone removal was postponed until the next procedure.

Stent migration

We defined stent migration in the present study as proximal migration of the stent into the bile duct.

Retrieval of migrated stents

We retrieved the migrated stents by using a basket catheter (4 or 8 wires), snare catheter, rat-toothed forceps, biopsy forceps, balloon catheter, or stent retriever.

Study items

We retrospectively examined the frequency of stent migration, and analyzed the patient factors [disease, whether endoscopic sphincterectomy (EST) was performed, location of bile duct stenosis, diameter of the bile duct] and stent factors (duration of stent placement, stent type, diameter and length). We also investigated the retrieval methods for migrated stents and their success rates.

Statistical analysis

Results were expressed as means or as a percentage of the total number of patients. The Mann-Whitney *U* test and the χ^2 test were used to compare differences between

Table 1 Diseases requiring bile duct stenting

Diseases	*n*	Migrated	Non migrated
Benign	227	12	215
Common bile duct stone	133	3	130
Chronic pancreatitis	62	4	58
Autoimmune pancreatitis	14	2	12
Trauma	8	1	7
Post operation	6	2	4
PSC	4	0	4
Malignant	169	2	167

PSC: Primary sclerosing cholangitis.

Table 2 Disease

Diseases	*n*	Migrated	Non migrated	*P* value
Benign	227	12	215	0.03
Malignant	169	2	167	
EST	263	11	252	0.400
Non EST	133	3	130	
Low	156	10	146	0.031
Hilar or middle	107	1	106	
Bile duct > 10 mm	154	11	143	0.023
Bile duct ≤ 10 mm	135	2	133	
≤ 1 m	263	4	259	0.007
> 1 m	133	10	123	

EST: Endoscopic sphincterectomy.

the two groups. $P < 0.05$ was considered to be significant. All analyses were performed using statistical software (Stat View Ver.5.0; SAS Institute, Cary, NC, United States).

RESULTS

Frequency of stent migration

The frequency of stent migration was 3.5% (14/396) (Table 1). Stent migration occurred in cases with common bile duct stones (3/190; 1.6%), malignant lesions (2/112; 1.8%), chronic pancreatitis (4/62; 6.5%), autoimmune pancreatitis (2/14; 14.3%), trauma (1/8; 12.5%), surgical complications (2/6; 33.3%) and PSC (0/4; 0%) (Table 1).

Patient factors

Disease (benign or malignant): In total, 12 of 14 cases (85.7%) of stent migration had benign disease. The overall number of cases with benign disease in this series was 227/396 (57.3%). The frequency of migration was significantly higher in cases with benign disease, such as chronic pancreatitis and autoimmune pancreatitis, when compared with cases of malignant disease ($P = 0.030$) (Figure 1, Table 2).

Cases undergoing EST: In total, 11 of 14 cases (78.6%) of stent migration had previously undergone EST. The overall number of cases who had undergone EST in this series was 263/396 (66.4%). The frequency of migration was not significantly higher in cases who had undergone EST when compared with cases who had not undergone EST ($P = 0.40$) (Table 2).

Location of bile duct stenosis: Among the cases with bile duct stenosis, except for cases with common bile duct stones, stenosis of the lower common bile duct was noted in 10 of the 11 cases (90.9%) with stent migration. Among the total number of cases with bile duct stenosis in the series, stenosis of the lower common bile duct was noted in 156 of 263 cases (59.3%). The frequency of migration was significantly higher in cases with stenosis of the lower common bile duct when compared with cases with hilar stenosis or stenosis of the middle common bile duct ($P = 0.031$) (Figure 1, Table 2).

Bile duct diameter: Among the cases of stenosis of the lower bile duct, except for those with hilar stenosis or stenosis of the middle bile duct, a bile duct diameter > 10 mm was noted in 11 of 13 cases (84.6%) of stent migration. The overall number of patients with a bile duct diameter > 10 mm was 154/289 (53.3%). The frequency of migration was significantly higher in cases with a bile duct diameter > 10 mm when compared with cases with a bile duct diameter ≤ 10 mm ($P = 0.023$) (Figure 1, Table 2).

Stent factors

Duration of stent placement: Stent placement duration > 1 mo was noted in 10 of 14 cases (71.4%) of stent migration. The number of patients with stent placement duration > 1 mo in the series was 133/396 (33.6%). The frequency of migration was significantly higher in cases with stent placement duration > 1 mo when compared with ≤ 1 mo ($P = 0.007$) (Figure 1, Table 2).

Stent shape: Straight-type stents were used in 11 of 14 cases (72.7%) of stent migration. The overall number of patients receiving straight-type stents was 133/396 (33.6%). The frequency of migration was significantly higher in cases with straight-type stents when compared with pig-tailed stents ($P < 0.001$) (Figure 1, Table 3).

Stent diameter: In total, 11 of 14 cases (78.6%) of stent migration received a 10-Fr stent. The overall number of patients who received a 10-Fr stent was 116/396 (29.3%).

Table 3 Stent shape, diameter and length

Stent	Factor	*n*	Migrated	Non migrated	*P* value
Stent shape	Pig-tail	263	3	260	< 0.001
	Straight	133	11	122	
Stent diameter	7 Fr	280	3	277	< 0.001
	10 Fr	116	11	105	
Stent length	5 cm	108	5	103	1.000
					0.530
	7 cm	270	8	262	2.000
					> 0.990
	9 cm	18	1	17	3.000
					0.450

5 cm *vs* 7 cm; 2.5 cm *vs* 9 cm; 3.7 cm *vs* 9 cm.

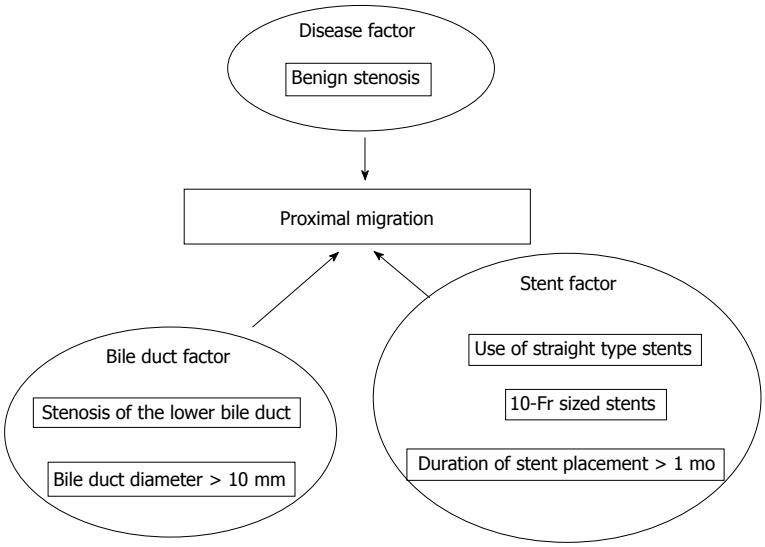

Figure 1 Potential risk factors. The potential risk factors for migration included bile duct stenosis secondary to benign disease (P = 0.030); stenosis of the lower bile duct (P = 0.031); bile duct diameter > 10 mm (P = 0.023); duration of stent placement > 1 mo (P = 0.007); use of straight-type stents (P < 0.001); and 10-Fr stents (P < 0.001).

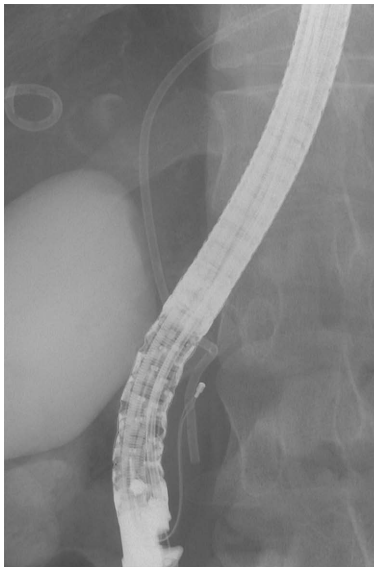

Figure 2 Grasping technique (Case 1). Examination was performed for hilus bile duct tumor. Pig-tailed-type 7-Fr stent migrated upon stent insertion. An opened eight-wired basket grasped the distal part of the stent. Stent retrieval was successful in Case 1.

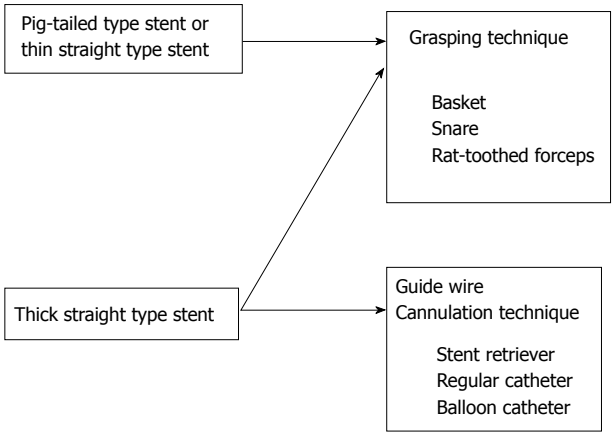

Figure 3 Retrieval methods for migrated stents.

The frequency of migration was significantly higher in cases with 10-Fr stents compared with 7-Fr stents (P < 0.001) (Figure 1, Table 3).

Stent length: A 5-, 7- and 9-cm stent was used in 5/14 (57.1%), 8/14 (57.1%) and 1/14 (7.1%) cases of stent migration, respectively. The overall number of patients who received a 5-, 7- and 9-cm stent was 108/396 (27.3%), 270/396 (68.2%) and 18/396 (4.5%), respectively. No significant differences in the frequency of migration were noted among the patients who received 5-, 7- and 9-cm stents (Table 3).

Retrieval methods for migrated stents, and their success rates

The grasping technique was used in eight of 14 (57.1%) cases of stent migration, and 60% of the cases had 7-Fr pig-tail stents. This is a technique of retrieval that is attempted by directly grasping the distal end of the stent with a basket (Figure 2), a snare, rat-toothed forceps or biopsy forceps. This method is effective for pig-tailed or thin straight stents (Figure 3). The cannulation technique was used in six of 14 (42.9%) cases of stent migration, and all cases had 10-Fr straight stents. This is a technique of retrieval that is attempted by connecting the distal end of the stent with a stent retriever, a balloon catheter, and a cannula (Figure 4) by using the guidewire passed through the lumen of the migrated stent. This method is effective for thick straight stents (Figure 3). Stent retrieval was successful in all cases.

DISCUSSION

Endoscopic biliary stenting using a tube stent is now a well-established therapy for biliary obstruction developing secondary to malignant or benign disease[1]. However, certain complications may develop with this technique and should be carefully considered. The complication rate for biliary stents reportedly ranges from 8% to

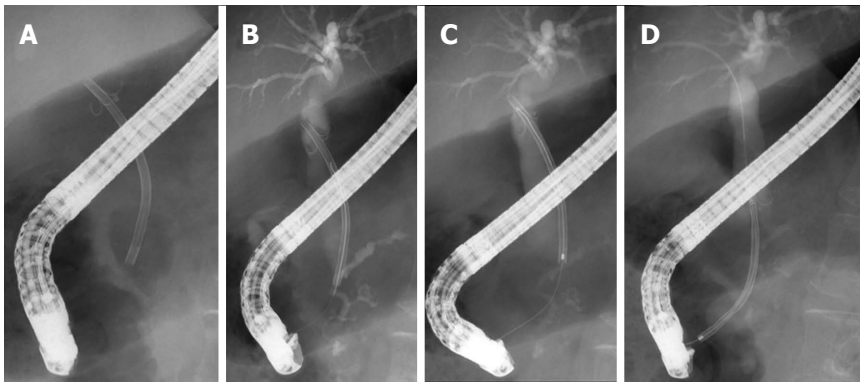

Figure 4 Cannulation technique (Case 2). A: Biliary drainage was performed for bile duct stenosis due to alcoholic chronic pancreatitis. Straight-type, 10-Fr stent migrated 6 mo after insertion; B: The guidewire was passed through the lumen of the migrated stent; the distal end of the migrated stent was connected with a cannula; C: Migrated stent was pulled, orienting the axis carefully; D: Stent retrieval was successful in Case 2.

10%[2-4], and the complications include cholangitis, cholecystitis, duodenal perforation, bleeding, pancreatitis, stent fracture, proximal stent migration, distal stent dislocation, and stent occlusion resulting in recurrent biliary obstruction[5-16]. Stent migration, a rare complication associated with biliary stenting[7,8,12], is a late event following endoscopic stenting. It occurs in 5%-10% of patients who undergo biliary stenting, and may involve proximal or distal migration[7,8,15,17,18,20,23-28]. Previous studies have reported that 3.1%-4.9% and 3%-6% of patients undergoing biliary stenting experience proximal and distal stent migration, respectively[5,7,17,18,24,25,29,30]. In the present study, the frequency of stent migration was 3.5%, which is consistent with previous studies.

Stent migration can lead to symptoms of biliary obstruction, therefore, it is important to determine the risk factors of stent migration[17,20,31]. However, thus far, the risk factors for biliary stent migration have yet to be clarified. Arhan *et al*[32] reported that proximal stent migration occurs in cases with cholangiocarcinoma, short stents, or stents with large diameters. Moreover, the placement of multiple stents and breaking of stents may cause stent migration. In addition to these risk factors, we also analyzed other possible risk factors including subtypes of both benign and malignant biliary disease; EST use; location of the stenosis; duration of stent placement; and stent shape, diameter, and length.

A previous study has indicated that stent displacement is less frequently noted in malignant than in benign biliary stenosis, which may be attributed to the tight covering of the stents by malignant tissue[32]. Similarly, in the present study, the frequency of migration was significantly higher in cases of benign than malignant disease (P = 0.030). One possible explanation for this phenomenon is that stenosis in benign disease is not as tight as that in malignant disease, which may be due to differences in the resolution of local inflammation. In cases of malignant stenosis, tumor growth may help to anchor the stent, thus preventing its migration. Arhan *et al*[32] reported that stent migration was observed less frequently in post-cholecystectomy strictures than in other benign biliary stenosis, except in cases of PSC[32]; the authors had indicated that the tightness of the fibrotic stricture could be a possible explanation for this finding. However, it was difficult to assess these hypotheses due to the low number of available cases in the present study.

Data on whether undergoing an EST before placement of a biliary stent affects the risk of migration are scarce. EST did not significantly affect the frequency of stent migration in previous studies[17,27,29,33]. Similarly, in the present study, the frequency of stent migration was not significantly higher in the cases who had undergone EST compared to those who had not undergone EST (P = 0.40). However, one study indicated a higher frequency of stent migration in patients who had undergone stent placement without prior EST compared to patients who had undergone stent placement after undergoing EST. The higher migration rate in these patients was primarily because of distal migration, whereas the incidence of proximal stent migration was not influenced by EST[34]. Thus, undergoing EST prior to stent placement cannot be considered as a proven risk factor for proximal stent migration.

With regard to stricture location, we generally believe that distal stent dislocation occurred more frequently in cases with stenosis of the upper bile duct, whereas proximal stent migration occurred more frequently in those with stenosis of the lower bile duct. In the present study, the frequency of migration was significantly higher in cases with stenosis of the lower common bile duct when compared with hilar stenosis or stenosis of the middle common bile duct (P = 0.031). However, in cases with stenosis of the lower common bile duct, it was unclear whether the migration was related to stent length.

We believe that a greater amount of space in the proximal bile duct is necessary for migration. Therefore, we hypothesized that the stent would be more likely to migrate proximally in cases with a larger bile duct diameter. In the present study, the frequency of migration was significantly higher in cases with a bile duct diameter > 10 mm compared with ≤ 10 mm (P = 0.023).

Moreover, in the present study, the frequency of migration was also significantly higher in cases with stent placement duration > 1 mo compared with ≤ 1 mo (P

= 0.007). A longer duration of stent placement increased the risk of migration. Therefore, we suggest that the stents should be exchanged or removed after a short placement interval.

Biliary stent design may also affect the risk for migration. With regard to stent shape, we used pig-tailed shaped and straight tube stents only in the present study. Straight tube stents have been modified with side flaps or barbs to decrease the risk of migration[35]. Pig-tailed shaped stents were also used to decrease the risk of migration. In the present study, the frequency of stent migration was significantly higher in cases with straight-type stents when compared with pig-tailed stents ($P < 0.001$). We noted that the presence of side flaps or barbs was not sufficient to prevent stent migration. The diameter and length of the stents may also be associated with the risk of migration[17]. With regard to stent diameter, in the present study, the frequency of migration was significantly higher in the cases with 10-Fr stents compared with 7-Fr stents ($P < 0.001$). We believe that, because stenotic lesions of the bile duct were already improved in cases in which thick stents (such as 10-Fr stents) were used, these cases were more likely to experience migration. With regard to stent length, in the present study, no significant differences in the frequency of migration were noted among the patients who had 5-, 7- and 9-cm stents. Arhan *et al*[32] have reported that shorter stents tend to migrate proximally, whereas longer stents tend to migrate distally, in cases of benign biliary stenosis. Moreover, longer stents in the bile duct are less likely to migrate because a longer portion is fixed in the common bile duct, thus limiting proximal movement[17]. I think that we should pay attention to using shorter and straight stents in high-risk patients. I recommend using pig-tailed type stent for those cases.

Most of the stents that have migrated proximally can be successfully retrieved indirectly, through stone extraction with a balloon catheter, or directly by using various grasping accessories. All the stents were successfully retrieved in the present study, therefore, none of the patients required surgery for stent retrieval. In patients with benign strictures, this favorable finding could be a result of the dilation of the stricture due to further stenting, thereby facilitating the retrieval of the proximally migrated stent.

In conclusion, the risk of stent migration is higher in benign than in malignant biliary stenosis. Proximal migration of biliary or pancreatic stents is an infrequent occurrence. Proximal stent migration was found to be closely associated with benign stenosis, stenosis in the lower common bile duct, bile duct diameter > 10 mm, stent placement duration > 1 mo, straight-type stents, and 10-Fr stents. The migrated stents can be extracted endoscopically, with a high degree of success, using a variety of techniques involving baskets or balloons.

COMMENTS

Background

Endoscopic biliary stenting using a tube stent is currently a well-established therapy for biliary obstruction developing secondary to malignant or benign disease. Endoscopic biliary stents may migrate proximally or distally as a late complication in 5%-10% of patients who have undergone biliary stenting. It is important to determine the factors influencing biliary tube stent migration. However, a few studies have reported on the risks of stent migration.

Research frontiers

In this study, the frequency of tube stent migration in the total patient population was 3.5%. The potential risk factors for migration included bile duct stenosis secondary to benign disease ($P = 0.030$); stenosis of the lower bile duct ($P = 0.031$); bile duct diameter > 10 mm ($P = 0.023$); duration of stent placement > 1 mo ($P = 0.007$); use of straight-type stents ($P < 0.001$); and 10-Fr stents ($P < 0.001$).

Innovations and breakthroughs

Retrieval of the migrated stents was successful in all cases. This paper describes the methods used for retrieval of migrated stents. The grasping technique, using a basket or snare, was effective for pig-tailed or thin and straight stents, whereas the guidewire cannulation technique was effective for thick and straight stents.

Applications

It is important to pay attention to the factors influencing biliary tube stent migration, and know the method for retrieval of migrated stents.

Terminology

Proximal stent migration is a rare complication associated with biliary stenting. Proximal migration means movement of stent distal end into common bile duct. Proximal stent migration can cause biliary obstruction and cholangitis, thus requiring retrieval or re-stenting.

Peer review

The potential risk factors for migration included bile duct stenosis secondary to benign disease; stenosis of the lower bile duct; bile duct diameter > 10 mm; duration of stent placement > 1 mo; use of straight-type stents; and 10-Fr stents. It is important to pay attention to these risk factors, and know the method for retrieval of migrated stents. These results are impressive.

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BRIEF ARTICLE

A new hemostatic clip for endoscopic surgery that can maintain blood flow after clipping

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Abstract

AIM: To develop a new hemostatic device for endoscopic surgery that can control the bleeding without completely occluding the bleeding vessel.

METHODS: A hemostatic clip and its applier that can stanch bleeding while maintaining blood flow through the clipped vessel was introduced, and the performance of the proposed clip was evaluated using *in vitro* and *in vivo* experiments.

RESULTS: During *in vitro* experiments, no leakage was found after clipping at cuts made in artificial vessels, and flow was maintained through the clipped artificial vessels. In experiments on rats, all the implanted clips occluded the target vessels successfully, and no bleeding or tissue damage was observed at the operative site after the rats were euthanized on postoperative day 7. In experiments on pigs, bleeding stopped immediately after partial clipping of a damaged vessel, and some amount of blood flow was consistently maintained through the clipped vessel after hemostasis.

CONCLUSION: We believe that the proposed hemostatic clip and clip applier can enhance patient safety during laparoscopic surgery.

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Key words: Vessel bleeding; Hemostasis; Hemostatic clip; Laparoscopic surgery; Endoscopic surgery

Core tip: This paper present a preclinical experimental study aimed at demonstrating the utility of a new clip designed to preserve blood flow while clipping a bleeding vessel. It can be a reasonable solution for partial large vessel injuries or avulsion injuries.

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INTRODUCTION

Most laparoscopic operations are performed using the limited 2-dimensional visual information about the operative site that is supplied by an endoscope; as a result, accidents due to the narrow field of vision and low depth perception frequently occur. One of the most common accidents is blood vessel injury caused by the misuse of cutting tools such as scissors, electrocautery, or harmonic scalpel[1]. Various hemostatic tactics can be applied to accidental vascular injuries that occur during laparoscopic surgery. These include the use of energy-based electrosurgical devices[2,3], insertion of hemostatic tubes

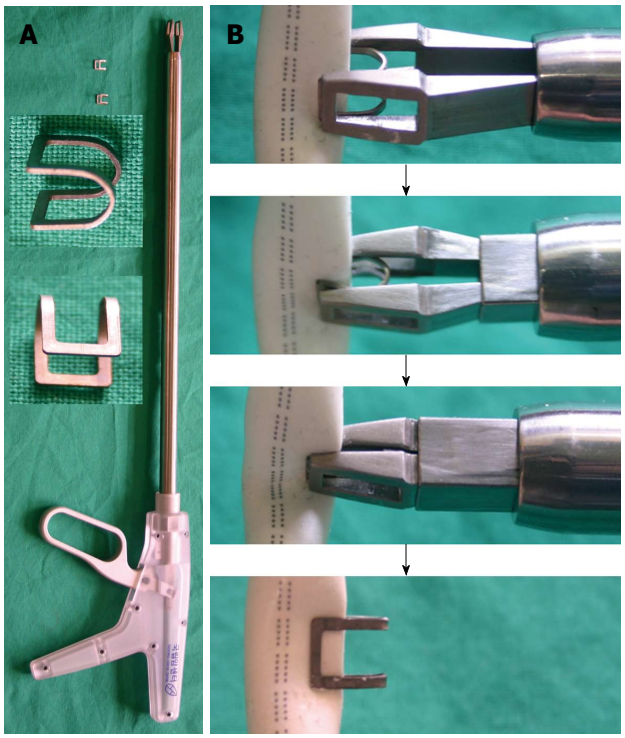

Figure 1 Proposed laparoscopic hemostatic clip and clip applicator. A: Shape of the clip and clip applier; B: Procedure for sealing a damaged vessel without fully occluding it.

or catheters[4,5], injection of hemostatic solution[6], use of hemostatic clips alone[7,8] or with epinephrine injection[9,10], injection of Histoacryl[11], or the use of topical hemostatic agents such as gelatins, collagens, thrombin and fibrin sealants, and synthetic glues[12-15].

However, energy-based vessel-sealing devices can cause thermal injuries that can result in neural damage and necrosis[16] or tissue distortions[17]. Additionally, when the damaged vessel cannot safely be completely occluded, it is difficult with conventional hemostatic clips to achieve hemostasis while maintaining blood flow through the damaged vessel after clipping[18].

In this article, we propose a new hemostatic clip and dedicated clip applier that can stanch bleeding from a damaged vessel during laparoscopic surgery without completely occluding the vessel and report on our *in vitro* and *in vivo* evaluation of their performance.

MATERIALS AND METHODS

Proposed laparoscopic hemostatic clip and clip applier

The proposed hemostatic clip and clip applier are shown in Figure 1A. The clip is 6 mm wide, 6 mm long, and 5 mm high, and the total length of the clip applier is 480 mm with a working length of 320 mm. When accidental vascular injury and bleeding occur during laparoscopic surgery, the clip is to be used as follows: (1) temporarily stop the main procedure; (2) grasp the bleeding vessel using laparoscopic forceps; (3) locate the clip applier so that the defect in the vessel is completely surrounded by the clip; (4) manipulate the handle of the clip applier to hold the hemostatic clip in place until the bleeding is stopped; and (5) resume the main procedure (Figure 1B).

In vitro evaluation

To evaluate the hemostatic performance of the clip in situations where it is important to maintain blood flow through the bleeding vessel, a closed-flow circuit consisting of a pneumatic pulsatile-flow pump and a Donovan-type mock circulatory system was assembled to simulate the main circulatory system of the human body, as shown in Figure 2A. The mock circulatory system and the sac of the pulsatile-flow pump were connected with 2 Tygon tubes with an internal diameter of 1/2 inch, to simulate an aorta and a vena cava. Then, the aortic tube was bifurcated to establish 2 flow paths for 1/2-inch and 1/4-inch artificial vessels, as shown in Figure 2B. Three hydraulic pressure sensors (PS9030VY™; Sensortechnics GmbH, Puchheim, Germany) and an ultrasonic flow meter (TS410; Transonic Systems Inc., Ithaca, NY, United States) were attached to the closed-flow circuit to observe the variations of hydraulic pressure and flow in accordance with the degree of vessel occlusion.

During the experiments, the average afterload pressure of the mock circulatory system was adjusted to 100 mmHg and the pumping rate of the pulsatile pump was fixed to 50 beats per minute to limit the maximal hydraulic pressure within the artificial vessel to $\leqslant$ 200 mmHg when the artificial vessel was completely occluded (average outflow of the hydraulic pump = 3.0 L/min). During experiments on the 1/4-inch artificial vessel, the tubes before and after the 1/2-inch artificial vessel were completely occluded using 2 clamping scissors and similarly, the tubes before and after the 1/4-inch artificial vessel were completely occluded during experiments on the 1/2-inch artificial vessel.

In vivo evaluation

To assess the stability and clinical feasibility of the clip, short-term animal experiments were performed using 4 rats (all males; average age = 3 mo) for full-occlusion tests and 2 pigs (all females; average age = 3 mo, average weight = 40 kg) for partial-occlusion tests. The rat experiments were performed at the Korean National Cancer Center, and the pig experiments were performed at the Yonsei University (Sinchon Severance Hospital). The experimental protocols were approved by the ethics committees for animal study of both medical institutions, and the studies were performed in strict compliance with the Korean Ministry of Health and Welfare Guidelines for the Care and Use of Research Animals.

RESULTS

Table 1 shows the measurements of the 3 pressure sensors during *in vitro* experiments lasting 3 h. The 1/4-inch artificial vessel was occluded 0%, 25%, 50%, and 75% using the proposed hemostatic clip and the 1/2-inch ar-

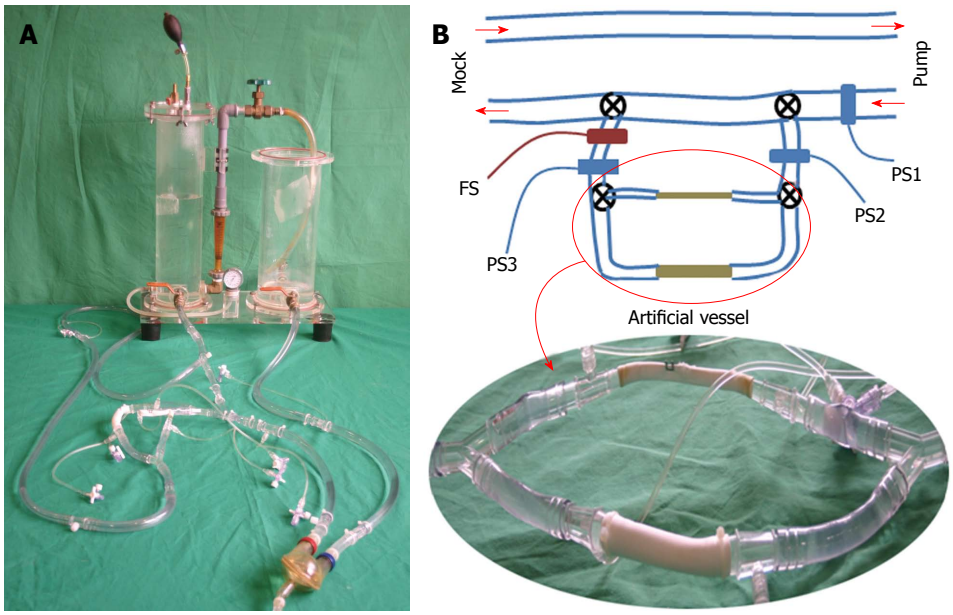

Figure 2 Setting for in vitro evaluation of the clip. A: Closed-flow loop including a pulsatile pump, a mock circulatory device, and 2 artificial vessels; B: 1/4-inch and 1/2-inch artificial vessels. PS: Pressure sensor; FS: Flow sensor.

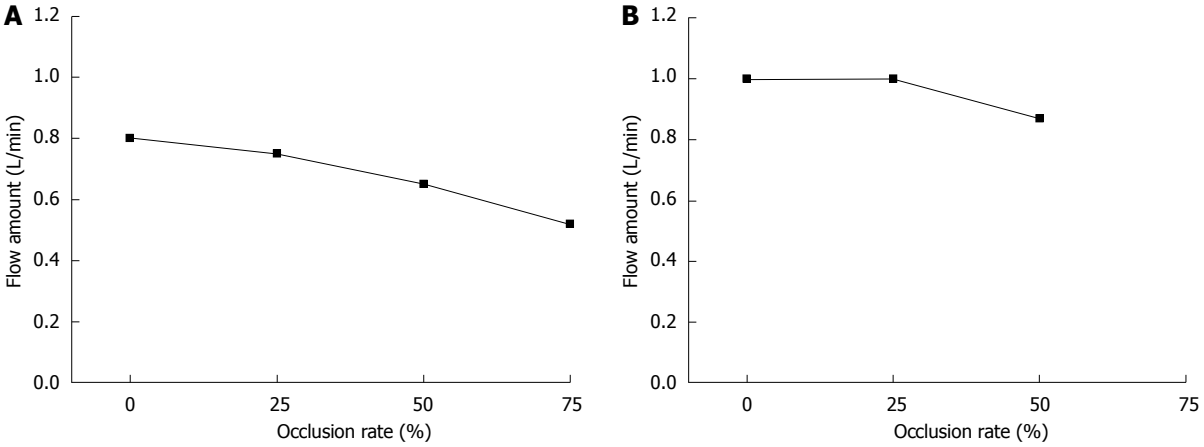

Figure 3 Flow variations according to the occlusion rate. A: 1/4-inch artificial vessel; B: 1/2-inch artificial vessel.

tificial vessel was occluded 0%, 25%, and 50%. Figure 3 shows the measurements of flow variation through the clipped artificial vessels. In all cases, no leakage at the cut area was found after clipping, and flow through the clipped artificial vessel was maintained after clipping the cut area. The average decreases in flow rates due to the clipping were 6.25% at 25% occlusion, 18.75% at 50% occlusion, and 35% at 75% occlusion for the 1/4-inch artificial vessel and 0% at 25% occlusion and 13% at 50% occlusion for the 1/2-inch artificial vessel.

Figure 4 demonstrates the procedures of the full-occlusion test. During the operation, each rat was laid on the operating table in a supine position; in 2 rats, the clip was implanted on the right iliac vein, in 1, on the inferior vena cava, and in 1, on the splenic artery and vein. The target vessels were completely occluded by the clip. The rats fully recovered from anesthesia within a few hours and were given food and water for 1 wk without any additional medical treatments. After the operation, 1 of the 4 clip-implanted rats died on postoperative day 3 and the remaining 3 lived for 1 wk. All of the surviving rats were euthanized on postoperative day 7 by anesthetic overdose. During autopsy, all of the implanted clips were seen to successfully occlude the target vessels and no bleeding or tissue damage was observed at the operative site.

Figure 5 demonstrates the procedures of the partial-occlusion test. Partial-occlusion tests were performed as laparoscopic surgeries. The target vessel (infra-hepatic inferior vena cava) was separated from the surrounding tissues, and then bleeding was intentionally induced on the surface of the target vessel using electrocautery. After the intentional injury, the armed clip applier was inserted through a trocar, and the bleeding site was occluded by the clip. During the experiment, the bleeding stopped immediately after clip implantation.

To assess blood flow through the clipped vessel, the

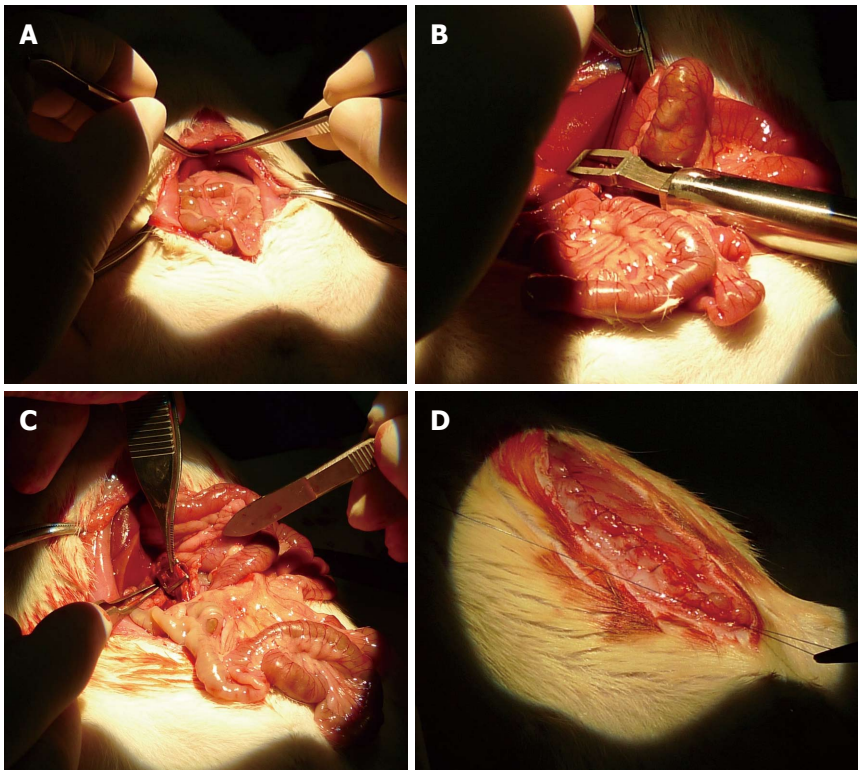

Figure 4 ***In vivo*** **experiments for full-occlusion testing.** A: The rat is supine on the operating table with the abdomen opened; B: The clip applier is positioned on the target vessel; C: The target vessel is clipped; D: The abdomen is sutured after clipping.

Table 1 Variation in pressure according to the occlusion rate (mean ± SD)

D	OR	PS1 (mmHg)	PS2 (mmHg)	PS3 (mmHg)
1/4	0%	149.57 ± 0.73	138.06 ± 0.59	129.83 ± 0.64
	25%	132.06 ± 0.80	123.15 ± 0.54	117.71 ± 0.82
	50%	149.85 ± 1.27	139.95 ± 1.20	138.05 ± 1.32
	75%	149.09 ± 0.56	135.18 ± 0.50	136.16 ± 0.57
1/2	0%	160.38 ± 1.22	152.42 ± 1.70	143.89 ± 2.45
	25%	124.20 ± 2.26	124.20 ± 2.26	125.62 ± 3.41
	50%	137.30 ± 1.46	130.25 ± 1.17	122.44 ± 1.40

D: Diameter (inch); PS: Pressure sensors in Figure 2B.

flow through the partially-clipped vessel was monitored using ultrasound (M-Turbo; Fujifilm Sonosite Inc., Tokyo, Japan). Because the ultrasonic probe utilized was not designed for laparoscopic use, the abdomen was opened and the probe was applied on the surface of the clipped vessel. Figure 6 shows the measurements of blood flow through the partially-clipped vessel. As is shown in the figure, some amount of blood flow was consistently maintained after clipping. After the measurements were taken, the pigs were euthanized by anesthetic overdose.

DISCUSSION

The clip and clip applier proposed in this article have a similar operating mechanism to the conventional over-the-scope clip[19,20] or the deep pistol-shaped clip applier[21]. The over-the-scope clip is primarily used for closing a perforation or fistula during laparoscopic surgery, but the proposed clip aims to immediately stanch bleeding from an injured blood vessels while maintaining blood flow through the clipped vessel. Additionally, the deep pistol-shaped clip applier aims to seal the bleeding of the venous channel at the intercavernous sinus during dural incision, but the proposed clip can cover a wider range of vascular injuries and therefore can minimize the number of clips needed to control bleeding from an injured vessel. Furthermore, unlike conventional hemostatic clips[22,23] that completely occlude blood vessels, the proposed clip can maintain a certain amount of blood flow through a clipped vessel.

Currently, the proposed clip is manufactured using biocompatible titanium; however, this presents the problem of postoperative incompatibility with magnetic resonance imaging[24]. To prevent this, the clip needs to be manufactured using non-metallic materials or non-magnetic metals (such as nitinol[25]) that have sufficient strength, durability, and biocompatibility. Additionally, applying the metallic clip to the bleeding vessel has a potential risk of tearing if the handle of the clip applier is tightened excessively by the surgeon, which might exacerbate the bleeding condition. This risk can be reduced by further improving the design of the clip applier to restrict the power transfer to the distal site of the clip applier, and surgeons undergoing additional preoperative training. Furthermore, sometimes, the clip may be applied on an unwanted area by mistake and should be removed immediately to reapply at the desired site. However, since

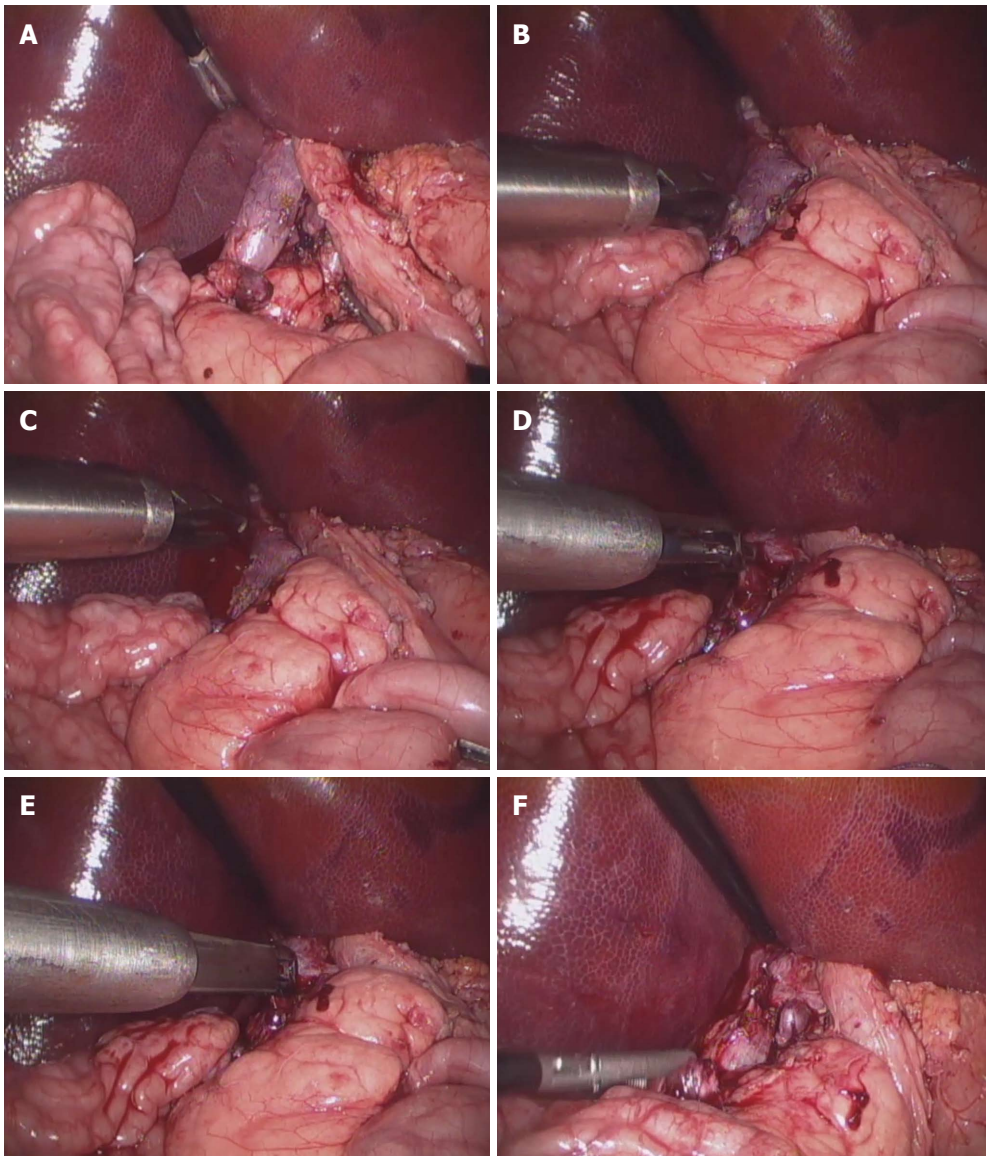

Figure 5 ***In vivo*** **experiments for partial-occlusion testing.** A: Separating the target vessel (infra-hepatic inferior vena cava) from surrounding tissues; B: Scratching the surface of the target vessel using electrocautery; C: Inducing intentional bleeding on the target vessel; D: Grabbing the bleeding site using the clip applier; E: Stanching the bleeding site on the target vessel by implanting the proposed hemostatic clip; F: Suctioning the blood around the target vessel after hemostasis.

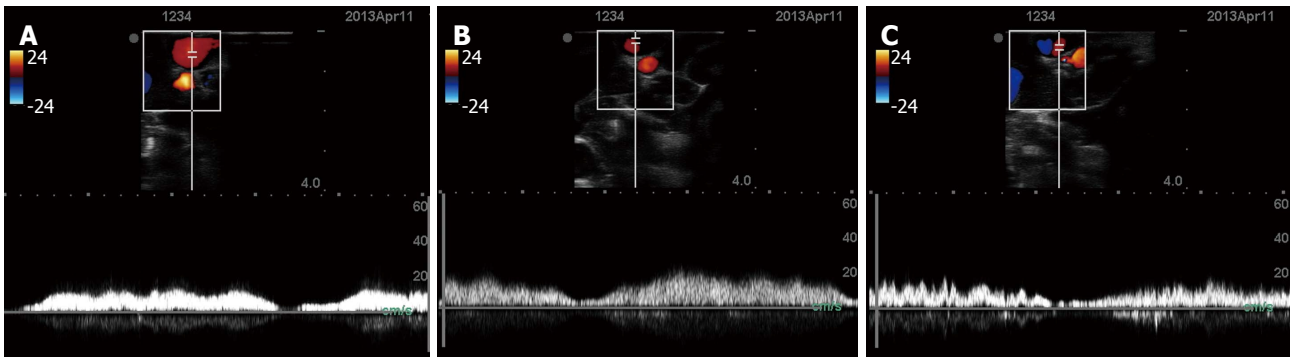

Figure 6 Measurements of blood flow through the clipped inferior vena cava using an ultrasonic Doppler machine. The probe was applied to the positions before (A), on (B), and after the clipped site (C), respectively. The Doppler signal was slightly increased at the clipped site because the diameter of the vessel decreased after clipping the bleeding site.

currently developed clip and clip applier do not have such clip-removal ability, we are in the process of implementing such ability by redesigning the structure of the clip and clip applier in the future. By adding such ability, it may even be possible to remove the clip after the bleeding site is sealed, using additional hemostatic agents and preventing clinical complications from the clip remaining in the body. Furthermore, if it is possible to manufacture the clip using bio-degradable materials such as absorbable hemostatic agents[26], the clip will degrade naturally after the bleeding is controlled.

Currently, the tip of the clip applier is straight, which makes it difficult to clip the bleeding vessel accurately in the perpendicular direction. To improve clinical feasibility, it will be necessary to redesign the clip applier to have 1 or 2 degrees of freedom at the distal end to be able to clip the bleeding vessel at various angles.

In this study, the sealing performance of the proposed clip was evaluated by *in vitro* and short-term animal experiments. Further studies are needed to investigate additional clinical concerns about the proposed hemostatic clip including intra-operative blood loss, surgical time, clip retention rate, and physiological and pathological effects on the vessel and surrounding tissues after long-term application of the clip[27-29] to verify its clinical feasibility.

In conclusion, we believe that the proposed hemostatic clip and clip applier have the potential to enhance patient safety during laparoscopic surgery.

COMMENTS

Background

Most laparoscopic operations are performed depending on the limited 2-D visual information and as a result, accidents such as blood vessel injury occur frequently. Currently, it is extremely difficult with conventional hemostatic clips to achieve hemostasis while maintaining blood flow through the clipped vessel.

Research frontiers

In the area of bleeding vessel hemostasis, the research hot spot is how to seal the bleeding site easily and quickly during operation.

Innovations and breakthroughs

Unlike conventional hemostatic clips, the hemostatic clip proposed in this paper can maintain a certain amount of blood flow through a clipped vessel.

Applications

The proposed hemostatic clip can cover a wider range of vascular injuries during laparoscopic surgeries.

Terminology

Hemostatic clip refers to the mechanical clip that seals or occludes the bleeding site during operation.

Peer review

The authors tried to do their best to establish the new sealed system to control intraoperative bleeding during the laparoscopic surgery. This new hemostatic clips and its applicator seemed to be function well, based on the *in vitro* and *in vivo* studies.

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BRIEF ARTICLE

Evaluation of specific fecal protein biochips for the diagnosis of colorectal cancer

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Abstract

AIM: To develop and initially test a potential fecal protein biochip for the screening of colorectal cancer (CRC).

METHODS: Fecal protein from 20 colorectal cancer patients and 20 healthy controls were extracted from all of the fecal samples and screened for proteomic differences using a Biotin label-based protein array. Candidate proteins were then verified by ELISA. Finally, we will select out the significant protein and a seven-target multiplex fecal protein biochip was generated and tested for 20 fecal samples to determine the effectiveness of the biochip on identifying CRC. And the value of the protein biochip would be discussed.

RESULTS: After tested by protein biochip of the fecal protein from 20 colorectal cancer patients and 20 healthy controls and levels of calprotectin, M2-pyruvatekinase, angiopoietin-2, fibroblast growth factor-23 (FGF-23), proteins of the matrix metalloproteinase, thrombopoietin (TPO) and interleukin-13 (IL-13) were significantly different between CRC and healthy controls. The sensitivity of all the seven proteins combined was 0.7, specificity was 0.4, and area under the receiver operating characteristics was 0.729. The most promising combinations of test proteins were FGF-23, TPO, and IL-13, reaching a sensitivity of 0.7 and a specificity of 0.7. The combination of FGF-23 and TPO scored highest with sensitivity of 0.7 and specificity of 0.8. Its mean that the combination of FGF-23 and TPO has the highest value for the diagnosis of CRC in our study.

CONCLUSION: A protein biochip composed of proteins found to be elevated in the feces of colorectal cancer patients has great potential as a noninvasive diagnostic for colorectal cancer. The addition of new protein biomarkers and technologies, as they are discovered, is an excellent avenue of future research.

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Key words: Protein biochip; Feces; Colorectal cancer; Fibroblast growth factor-23; Thrombopoietin

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INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide. About 1 million CRC cases oc-

cur each year, of which 500000 people will die[1]. There are multiple stages along the pathological progression of CRC. The progression occurs over 7-10 years from normal mucosa through adenoma to cancer[2]. Unfortunately, about 30%-50% of patients have metastases at the time of diagnosis; the 5-year survival rate is less than 10%[1]. In contrast, the 5-year survival of patients who are in Dukes A with surgical resection is more than 90%[3]. Therefore, it is imperative to detect CRC at an early stage in order to reduce mortality and morbidity.

There are many methods to detect CRC. Non-invasive methods include fecal occult blood test (FOBT), fecal immunochemical test (FIT), computer tomography (CT) colonoscopy, magnetic resonance imaging (MRI) colonoscopy, and barium enema. More invasive methods include flexible sigmoidoscopy and colonoscopy. FOBT, which is low-cost, simple, and painless, is the most widely used method. However, it has a high incidence of false positive and false negative results, as well as low specificity. The flexible sigmoidoscopy procedure can only detect cancer within the sigmoid colon and rectum[4]. Colonoscopy is considered the gold standard in the diagnosis of CRC[5], but its application is limited because of its high cost, invasiveness, patient discomfort during preparation, and complications, such as hemorrhage, intestinal perforation, and myocardial infarction.

As the normal biological function of colorectal epithelial cells becomes damaged, changes in oncogenes and tumor-suppressor genes result in mutations at the DNA, RNA, and protein levels[5]. Therefore, it may possible to develop a new method that detects these molecular changes in blood or feces; carcinoembryonic antigen (CEA) is an example of the earliest application of this strategy[6]. A detection method that uses fecal material has the advantages of being simple, flexible, and non-invasive, with no need for bowel preparation, and may reflect pathologies involving the whole gut. Molecules found in the feces include DNA, RNA, and protein. Detection of mutations in the genes encoding *KRAS*, *APC*, *BRAF*, and *TP53* have been proposed recently, but the sensitivity is too low[5] and it is a high cost, complex operation. Proteins, in contrast, are more suitable for such a large-scale screen, because of their stability, high sensitivity, and operation simplicity[7]. Therefore, fecal detection is a very promising method for the detection of biomarkers in the feces of CRC patients.

In this study, we collected fecal specimens from CRC patients and healthy controls, and used protein biochip methods to measure several significant proteins chosen from the current literature. Finally, we verified the value of them as combined CRC biomarkers in the diagnosis of CRC by protein biochip.

MATERIALS AND METHODS

Patients and healthy controls

A total of 20 CRC patients (10 men and 10 women) and 20 cases of healthy controls (14 men and 6 women) were collected. The average patient age was 67.5 years (range: 46-88 years). Within the CRC group, diagnoses were all confirmed as CRC by pathology from a colonoscopy biopsy. For both groups, patients were excluded if they had any one of the following: long-term history of irritable bowel disease (IBD); ulcerative colitis (UC); intestinal polyp; chronic constipation; diarrhea; hereditary intestinal polyposis syndrome and intestinal surgery; any diseases of the respiratory, urinary, cardiovascular, and nervous systems; or other diseases of the digestive system excluding the gastrointestinal tract. Patients were instructed to eat a normal protein diet for 1-2 wk prior to electronic colonoscopy. Information about the demographics of all test subjects is detailed in Table 1.

Table 1 Information of colorectal cancer patients and healthy controls *n* (%)

	Colorectal cancer (*n* = 20)	Healthy controls (*n* = 20)
Gender		
Male	10 (50)	14 (70)
Female	10 (50)	6 (30)
Age group (yr)		
46-55	5 (25)	5 (25)
56-65	3 (15)	4 (20)
66-75	6 (30)	7 (35)
⩾ 75	6 (30)	4 (20)
Location of tumor		
Ascending colon	6 (30)	-
Transverse colon	1 (5)	-
Descending colon	6 (30)	-
Rectum	7 (35)	-
Tumor stage (TNM)		
Ⅰ	2 (10)	-
Ⅱ	7 (35)	-
Ⅲ	9 (45)	-
Ⅳ	2 (10)	-

Feces collection and protein extraction

All stool specimens were collected 1-2 wk before surgery. Avoiding urine or water pollution, samples were collected, cut to 8.0 cm^3 in size (2.0 cm × 2.0 cm × 2.0 cm), and immediately preserved in -80 ℃ for at least 30 min. For each sample, 0.1 g feces weighed precisely, added to 0.4 mL RIPA lysis buffer, vortexed for 1 min alternating with a 5-min ice bath until no visible feces granules remained, and then centrifuged. The supernatant fraction was collected and put on ice for about 60 min, centrifuged for 30 min at 10000 rpm at 4 ℃, and then the supernatant fraction was collected again. The final supernatant was stored at -80 ℃. The samples of CRC were labeled as C1-C20; the healthy controls were labeled as N1-N20.

Protein concentration detection

The protein concentration was measured first by spectrophotometry using a NanoDrop ND-2000 (Thermo Fisher, United States), and then by bicinchoninic acid (BCA) using a standard protocol. The protein concentra-

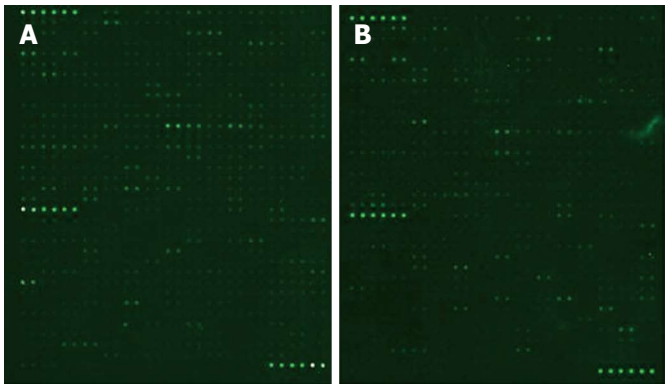

Figure 1 Example images of protein biochip scans of colorectal cancer and healthy control samples. A: Normal control group; B: Colorectal cancer group.

Table 2 Data of the first five proteins after normalization

Name	C-Nor	N-Nor	C/N
Angiopoietin-2	263.60	6.89	38.27
MMP-10	223.58	10.14	22.05
FGF-23	345.69	22.60	15.3
Thrombopoietin	229.29	18.03	12.72
IL-13	243.38	21.05	11.56

N: Normal control group; C: Colorectal cancer group; MMP: Proteins of the matrix metalloproteinase; FGF: fibroblast growth factor-23; IL-13: Interleukin-13.

tion was tested by colorimetric determination under 562 nm and compared to a standard curve of bovine serum albumin.

Protein biochip screen and test

Fecal samples were analyzed by RayBio Biotin Label-based Human Antibody Array1 (507 Human Proteins), using Raybiotech kits. Samples were dialyzed, biotin labeled, dried, blocked, and then incubated in Antibody Array. Raw data were collected by fluorescence detection (GenePix 4000 b, Axon Instruments, United States), using the GenePix Pro 6.0 software (Axon Instruments, United States).

Enzyme linked immunosorbent assay test

After screening by protein biochip and literature review, we chose seven proteins for enzyme linked immunosorbent assay (ELISA) analysis. For each protein, 10 μL of sample were tested by ELISA using the manufacture's protocol (Labsystems Multiskan MS, Finland). A standard curve was drawn with the concentration of the standard as abscissa, the *A* value as ordinate.

Ethics

This study was approved by the ethics committee of The 5th People's Hospital of Shanghai, Fudan University. All the patients in the study signed consent forms before participating.

Statistical analysis

The statistical differences concentration of selected proteins between CRC and healthy controls were determined using the *t* test. A *P* value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 16.0 software.

RESULTS

To determine the proteomic differences in fecal matter of CRC and healthy control samples, we screened fecal samples using a biotin label-based human antibody array 1. The results are shown as scanned images (Figure 1). The raw data were read using GenePix Pro 6.0 software, and the normalized results are shown in Table 2. Normalization was calculated by the size ratio of CRC group (C)/healthy controls (N). Initially, we found five proteins with maximum differences between the cohorts: angiogenin 2 (Ang-2), matrix metallopeptidase 10 (MMP-10), fibroblast growth factor-23 (FGF-23), thrombopoietin (TPO), and interleukin 13 (IL-13). We decided to use these five candidate proteins for further analysis.

Through a literature search, we uncovered two additional candidate proteins for analysis: calprotectin and pyruvate kinase isoenzyme type M2 (M2-PK). We measured all seven proteins in the fecal samples using ELISA. During sample processing, we lost C5 from the CRC group and N9 and N17 from the control group. Thus, the final sample sizes were: CRC, $n = 19$; Healthy, $n = 18$. The concentrations of the seven proteins in CRC group and healthy controls are shown in Figure 2A and B. After receiver operating characteristic curve (ROC) analysis, the cut off and area under the curve (AUC), sensitivity and specificity were calculated, as shown in Figure 3A and Table 3. The levels of calprotectin, M2-PK, Ang-2, FGF-23, MMP-10, TPO, and IL-13 were significantly higher in CRC cases compared with the controls.

Calprotectin, sensitivity 1, specificity 1, average concentration of group C was 179.12 IU/mL, N was 116.08 IU/mL; M2-PK, sensitivity 0.895, specificity 0.889, average concentration of group C was 141.15 IU/mL, N was 100.19 IU/mL; Ang-2, sensitivity 1, specificity 1, average concentration of group C was 86.46 IU/mL, N was 55.85 IU/mL; MMP-10, sensitivity 0.895, specificity 1, average concentration of group C was 518.37 IU/mL, N was 348.61 IU/mL; FGF-23, sensitivity 1, specificity

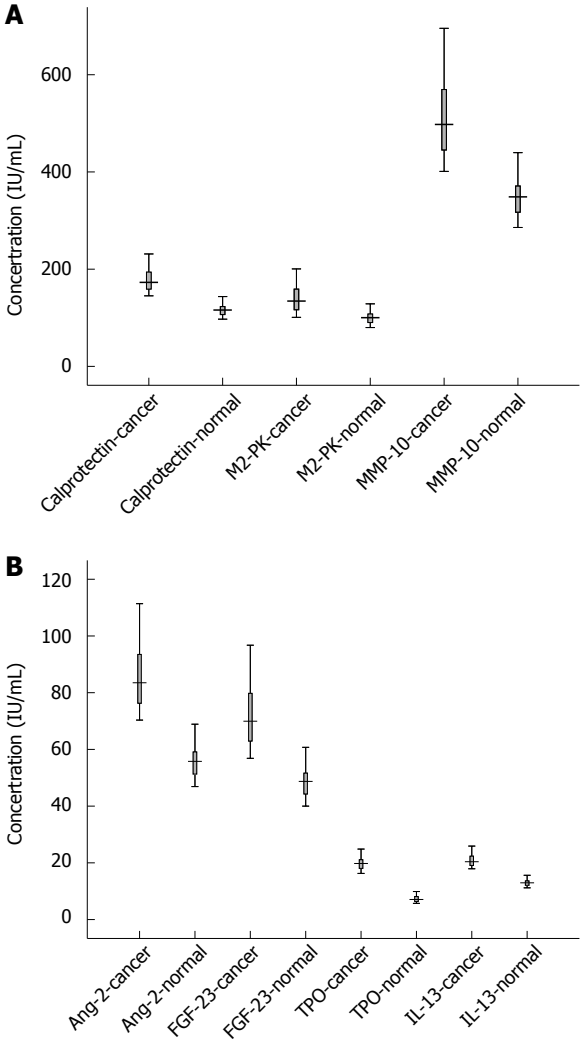

Figure 2 Median and interquartile range of proteins concentrations among colorectal cancer patients and healthy controls. A: Calprotectin, M2-pyruvatekinase (M2-PK), proteins of the matrix metalloproteinase (MMP-10); B: Angiopoietin-2 (Ang-2), fibroblast growth factor-23 (FGF-23), thrombopoietin (TPO) and interleukin-13 (IL-13).

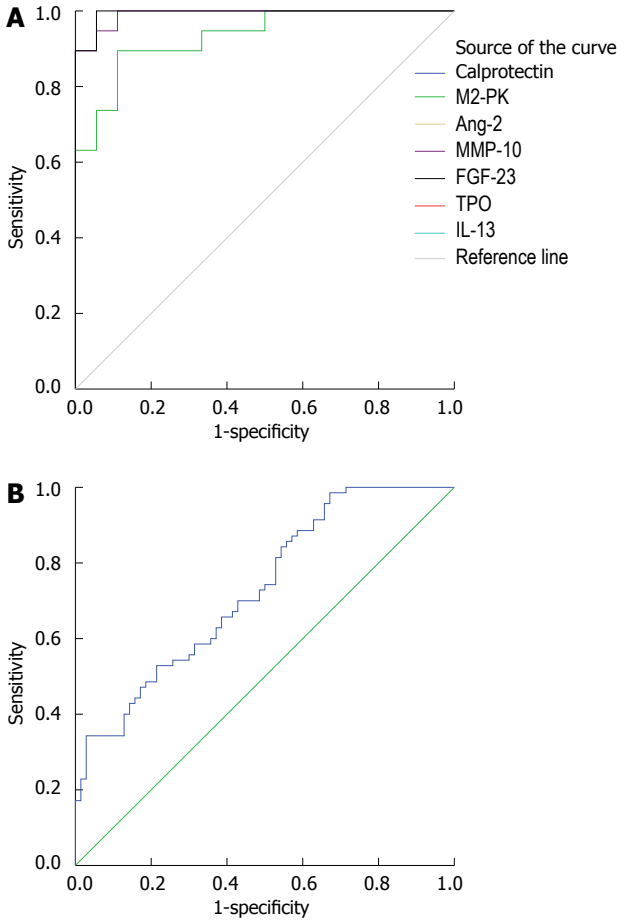

Figure 3 Receiver operating curve for the seven fecal protein. A: Receiver operating curve (ROC) for the seven fecal protein in detecting patients with colorectal cancer (CRC, n = 19) and healthy controls (n = 18). Calprotectin, angiopoietin-2 (Ang-2), thrombopoietin (TPO), interleukin-13 (IL-13): area under the curve (AUC) = 1; M2-pyruvatekinase (M2-PK): AUC = 0.933; matrix metalloproteinase (MMP-10): AUC = 0.991; fibroblast growth factor-23 (FGF-23): AUC = 0.994; B: ROC for the seven fecal protein combined in detecting patients with CRC (n = 10) and healthy controls (n = 10). Sensitivity= 0.7, specificity = 0.4. AUC = 0.729.

0.944, average concentration of group C was 72.84 IU/mL, N was 48.55 IU/mL; TPO, sensitivity 1, specificity 1, average concentration of group C was 19.76 IU/mL, N was 7.39 IU/mL; IL-13, sensitivity 1, specificity 1, average concentration of group C was 21.01 IU/mL, N was 12.99 IU/mL. After T-test and P values are calculated by SPSS16.0, $P < 0.05$, Table 4.

Finally, a protein biochip was made composed of the seven proteins. We selected 20 samples of 10 cases of CRC and 10 cases of healthy controls for retesting. The results are shown in Figure 3B. For the seven protein combined, the AUC = 0.729, the sensitivity was 0.7, and the specificity was 0.4. We also examined the sensitivity and specificity for select subsets of the seven proteins. For a subset containing FGF-23, IL-13, and TPO, the sensitivity was 0.7 and the specificity was 0.7. For a subset containing FGF-23 and TPO only, the sensitivity was 0.7 and the specificity was 0.8, making this the best combination for CRC screening. We also analyzed the correlation of the seven proteins with age of patients, tumor location (ascending colon, transverse colon, descending colon, or rectum), and tumor stage (Dukes stage or TNM stage) by t test; there were no statically significant correlations. Thus, the abundance of these seven proteins is independent of patient age, tumor location, and tumor stage.

DISCUSSION

There is an ongoing search for new methods and markers for the diagnosis of CRC. Many studies of attempted proteomics on CRC samples, but the results lack consistency for a number of possible reasons. First, there is always heterogeneity and variation in the experimental material, such as a varied patient population and different genetic and environmental factors, which can lead to different results. Second, the use of different research methods, such as 2D-polyacrylamide gel electrophoresis

Table 3 Information of area under the curve and 95%CI of the seven proteins

Test result variable(s)	Area	SE	Asymptotic sig	Asymptotic 95%CI	
				Lower bound	Upper bound
Calprotectin	1.000	0.000	0.000	1.000	1.000
M2-PK	0.933	0.039	0.000	0.856	1.009
Ang-2	1.000	0.000	0.000	1.000	1.000
MMP-10	0.991	0.010	0.000	0.972	1.011
FGF-23	0.994	0.008	0.000	0.979	1.010
TPO	1.000	0.000	0.000	1.000	1.000
IL-13	1.000	0.000	0.000	1.000	1.000

M2-PK: M2-pyruvatekinase; Ang-2: Angiopoietin-2; MMP-10: Proteins of the matrix metalloproteinase-10; TPO: Thrombopoietin; IL-13: Interleukin-13; FGF-23: Fibroblast growth factor-23.

Table 4 Cutoff value, 95%CI, *P* value of the protein concentration

Name	SE	mean	95%CI	Cutoff value	*P* value
Calprotectin-C	5.725	179.12	167.09-191.14	144.39	0.00
Calprotectin-N	2.906	116.08	109.95-122.21		
M2-PK-C	1.516	141.15	137.97-144.34	114.49	0.00
M2-PK-N	0.725	100.19	98.66-101.72		
Ang-2-C	0.627	86.46	85.14-87.78	69.62	0.00
Ang-2-N	0.324	55.85	55.16-56.53		
MMP-10-C	4.505	518.37	508.91-527.84	440.41	0.00
MMP-10-N	2.267	348.61	343.82-353.89		
FGF-23-C	0.607	72.84	71.56-74.11	56.33	0.00
FGF-23-N	0.304	48.55	47.91-49.19		
TPO-C	0.118	19.76	19.51-20.01	13.08	0.00
TPO-N	0.069	7.39	7.24-7.53		
IL-13-C	0.122	21.01	20.76-21.27	16.76	0.00
IL-13-N	0.064	12.99	12.85-13.12		

N: Normal control group; C: Colorectal cancer group; M2-PK: M2-pyruvatekinase; Ang-2: Angiopoietin-2; MMP-10: Proteins of the matrix metalloproteinase-10; TPO: Thrombopoietin; IL-13: Interleukin-13; FGF-23: Fibroblast growth factor-23.

and mass spectrometry, hinders the ability to properly compare results. Another factor is the lack of detailed experimental methods from independent researchers, thus direct comparisons cannot be made.

In contrast, there are several advantages to using stool specimens and protein biochip technology. First of all, the use of feces is noninvasive, resulting in a good patient compliance rate. Second, comparing abundance of a small set of proteins has shown to have relatively high sensitivity and specificity. This approach overcomes the disadvantage of diagnosing cancers using a single molecular marker. Third, the method is simple, fast and convenient. Fourth, the amount of fecal sample does not limit the analysis[8,9]. Finally, it shows that the abundance of tumor-specific proteins in feces is significantly high compared with blood; this sample type and can reflect the full length of the colorectum[1].

There are several types of proteins found in feces, including products of digestion, and cell secretion, shedding from the lumen, and bacteria. Protein from food has no specificity in stool specimens; however, in this study patients were requested to limit their intake of high-protein foods before sample collection, especially animal protein. In feces, bacteria constitute nearly half of the total sample weight, excluding water. By centrifuging the samples, these proteins were cleared completely[1]. Proteins that come from lumen can be roughly classified into three categories: leakage, secretion, and exfoliation[10]. Due to dysfunction of the blood and lymphatic vessels of tumor, certain molecules leaked into the feces. However, this is not a consistent or continuous process, and it can also occur with tumor lesions, thus it lacks sensitivity and specificity. The most typical example in this case is hemoglobin[11]. Many other molecules are secreted by the epithelial cells of the colorectal lumen. Cancer cells can secrete unusual glycosylated proteins, such as mucin[12,13], CEA, and mini chromosome maintenance proteins (MCM2). These proteins can be detected by specific antibodies, but the intestinal bacteria secrete proteases and other glycosidic enzymes that can interfere with this process. Still other exfoliated molecules are released into the lumen during apoptosis of epithelial cells; they often come directly from the tumor itself. As a result, they have a high specificity for the presence of tumors. The renewal speed of tumor tissue is usually higher than that of normal tissue, therefore these molecules are even more likely to be found. Tumor sources of protein or peptide sequence are very stable in the feces, such as M2-PK, DAF, CEA, *etc*., and have high specificity[10].

In this study, calprotectin, M2-PK, Ang-2, MMP-10, FGF-23, TPO and IL-13 were identified and verified as statistically significant tumor markers. Every molecule was analyzed individually. The sensitivity and specificity of calprotectin, Ang-2, TPO, and IL-13 are all highest, with a value of 1. This result may have been because of the small sample size. Calprotectin, a 36.5 kDa zinc/calcium-binding protein, is made up of three polypeptide chains. calprotectin mainly comes from neutrophils[14]. CRC is tightly linked to acute inflammation reactions[15], therefore it was reasonable to test the levels of calprotectin as a marker of CRC[16]. Calprotectin is stable in feces, and small samples can be measured reliably. Tibble *et al*[16] found that the sensitivity and specificity of calprotectin are 79% and 72%, respectively. In the same study, concentration of FOBT was found to have no correlation with the tumor location or Dukes staging; the same results were found in this experiment.

M2-PK, an important enzyme for the metabolism of tumor cells, is a dimer of pyruvate kinase isoenzyme and target of many oncogenes. Hardt *et al*[17,18] and Ayling *et al*[19] reported that M2-PK can be detected in feces of CRC patients by ELISA. Hardt *et al*[18] found that M2-PK concentrations in feces were significantly higher in the 60 cases of CRC than the control group by ELISA; the sensitivity was 73%, specificity was 78%, and the concentration of M2-PK is closely related to TNM and Dukes staging. Because of the high sensitivity and specificity of M2-PK, it has high potential as a biomarker of CRC.

Ang-2, the Tie-2 receptor ligand of tyrosine specificity in endothelial cells, can disrupt cellular stability to induce angiogenesis[20]. Ahmad *et al*[21] detected the expression of Ang-1 and Ang-2 by RT-PCR and double staining fluorescence immunoassay in CRC cell lines, as well as a CRC resection specimen and normal mucosa. The results showed that Ang-2 was not only expressed in normal cells, it was also highly expressed in colon cancer cells. These results indicate that Ang-2 may be the promoting factor in tumor angiogenesis.

FGF-23, which is 32 kDa, it is secreted by bone cells or osteoblasts. Its target organ is the kidney, and its main function is to regulate phosphate and vitamin D metabolism. Leaf *et al*[22] found the levels of FGF-23 were higher than normal in one patient with colonic carcinoma, suggesting a correlation between high FGF-23 levels and CRC[22,23]. MMP-10 is a member of the zinc-dependent family of endopeptidases. It can decompose the extracellular matrix and break the blood-brain barrier. Because the reconstruction of the extracellular matrix is related to tumor metastasis and invasion, MMP-10 is considered to be related to these events. Asano *et al*[24] found the expression of MMP-10 to be increased in 112 cases of colorectal tumor tissues.

IL-13 is a cytokine which has anti-inflammatory and immunomodulatory effects. Its overexpression in cancer tissue is associated with tumor infiltration, apoptosis suppression, tumor metastasis, and lower mucin calcium[25]. Formentini *et al*[26] investigated the expression of IL-13R in epithelial cells of ulcerative colitis, colonic cancer and control patients. They found that IL-13R levels were significantly higher in colonic cancer samples than in the control and ulcerative colitis groups. TPO is a cytokine produced mainly by the liver, but also by the kidneys and other organs. In the process of tumorigenesis, blood coagulation dysfunction, platelet activation, increased number of platelet and thrombosis are common. Dymicka-Piekarska *et al*[27] found that TPO levels in a set of 38 cases of CRC was significantly higher than that of a control group of 35 individuals. Furthermore the concentration was higher in stage Ⅲ tumors.

In this study, the concentration of all seven of these protein molecules was significantly higher in the CRC patients than the healthy controls, consistent with other research results. This result further validates the relationship between these seven proteins and CRC. Additionally, a protein biochip was used to combine measurements for all proteins simultaneously. With the seven protein molecules combined, sensitivity was 0.7 and specificity was 0.4. By combining FGF-23, IL-13, and TPO, the sensitivity was 0.7 and specificity was 0.7. If FGF-23 and TPO only were considered, the sensitivity was 0.7 and specificity was 0.8. Hence, FGF-23 and TPO is the best combination found in this study to accurately identify the presence of tumors from a fecal sample of the patient. We also analyzed the relationship between the seven protein levels and patient age, tumor location (ascending colon, transverse colon, descending colon, and rectum), and tumor staging (Dukes staging and TNM staging) by *t* test. Although the test was limited by sample size and a non-normal distribution, the results were not significant. In this study, the diagnosis value of a single molecular maker is comparable with other studies. However, due to the different sample size and research methods, the results also have slight differences. Studies using feces as a specimen source to discover protein biomarkers for CRC is scarce. Moreover, it is rarer to see this strategy combined with the protein biochip approach. Stefanie Bunger detected CEA, IL-8, vascular endothelial growth factor, S100A11, macrophage colony-stimulating factor, C3adesArg, CD26 in the blood of patient using a protein biochip approch, and the best combinations in this study were (1) CEA with IL-8; and (2) CEA with C-reactive protein; the sensitivity/specificity were 37%/83% and 35%/81%, respectively. This study provided a basis for the successful application of protein chips in the aid of CRC diagnosis. However, this study used blood samples as its specimen, but stool specimens may have additional advantages.

In our study, the sample size is small, so additional samples would afford increased confidence in our findings. The use of proteins as molecular markers may also have its shortcomings. First, proteins could be decomposed easily by proteases, leading to a false negative result. Second, there is no method to amplify protein as there is for DNA or RNA, thus limiting the application. Thirdly, changes in the phenotype of the tumor occur at the genomic level first, rather than the protein sequence[28]. Thus, protein molecules used as biomarkers need further in-depth study before they can be used widely for cancer screening.

In summary, we found seven proteins whose abundance is significantly higher in the feces of CRC compared with healthy controls. The strongest test combination is FGF-23 and TPO, with sensitivity of 0.7 and specificity of 0.8. These proteins did not correlate in any way to patient age, tumor stage, or location. We have shown that protein biochips have potential to be used as a noninvasive screening method for CRC. Additional protein biomarkers and optimized technology are areas for future research to develop this new diagnostic technique. With the discovery of new protein molecules and progress of diagnosis technology, the value of this method for CRC diagnosis will be subjected to further improvement and development.

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COMMENTS

Background

Colorectal cancer (CRC) is the second leading cause of cancer death worldwide. Fecal occult blood test and others have a high incidence of false positive and false negative results, as well as low specificity. Proteins, using fecal

material have the advantages of being simple, flexible, and non-invasive, with no need for bowel preparation, and may reflect pathologies involving the whole gut, are more suitable for such a large-scale screen, because of their stability, high sensitivity, and operation simplicity. Therefore, fecal detection is a very promising method for the detection of biomarkers in the feces of CRC patients.

Research frontiers

There are many researches in the finding out the biomarkers of CRC. DNA, RNA and protein are the most hotspots in this field. However, single molecular of which is deeply researched. And most of the single molecular biomarker in blood had a low sensitivity and specificity, especially there are less research of the fecal molecular biomarkers.

Innovations and breakthroughs

Fecal biomarker is a new method for cancer research. The aim of this study is not only to evaluation the value of specific fecal protein biochips, but also the authors wanted to find out new fecal molecular biomarkers for the diagnosis of CRC. It is significant for the diagnosis and treatment of CRC. The study is a research paper, and in the next period we will continue to focus on the specific fecal protein and the mechanism of them.

Applications

The study results suggest that the combination of fibroblast growth factor-23 (FGF-23) and thrombopoietin (TPO) by protein biochip method has a high value for the diagnosis of CRC and it may be used in clinical in the future.

Terminology

There is an ongoing search for new methods and markers for the diagnosis of CRC. Stool specimens and protein biochip technology is noninvasive, simple, fast and convenient, resulting in a good patient compliance rate and overcomes the disadvantage of diagnosing cancers using a single molecular marker. Proteins that come from lumen can be roughly classified into three categories: leakage, secretion, and exfoliation.

Peer review

This is a good retrospective study in which the authors analyzed the value of the protein biochip in the diagnosis of CRC. The results are valuable and suggest that the combination of FGF-23 and TPO by protein biochip method has a high value for the diagnosis of CRC. It is also need to be have a deep research in the future.

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BRIEF ARTICLE

Endoscopic ultrasonography for staging of T1a and T1b esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the accuracy of Endoscopic ultrasound (EUS) in staging and sub-staging T1a and T1b esophageal squamous cell carcinoma (ESCC).

METHODS: A retrospective analysis involving 72 patients with pathologically confirmed T1a or T1b ESCC, was undertaken between January 2005 and December 2011 in Sun Yat-sen University Cancer Center. The accuracy and efficiency of EUS for detecting stages T1a and T1b ESCC were examined.

RESULTS: The overall accuracy of EUS for detecting stage T1a or T1b ESCC was 70.8% (51/72), and the sensitivity was 74.3%. 77.8% (7/9) of lesions originated in the upper thoracic region, 73.1% (38/52) in the mid-thoracic region and 72.7% (8/11) in the lower thoracic region. Multivariate analysis revealed that the diagnostic accuracy of EUS was closely related to lesion length (F = 4.984, P = 0.029).

CONCLUSION: EUS demonstrated median degree of accuracy for distinguishing between stages T1a and T1b ESCC. Therefore, it is necessary to improve EUS for staging early ESCC.

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Key words: Endoscopic ultrasound; Esophageal cancer; Squamous carcinoma; Early cancer; Stage

Core tip: Endoscopic ultrasound (EUS) is the best option for staging early esophageal squamous cell carcinoma (ESCC). However, the accuracy of EUS for staging ESCC is not good and diverse. There is no other report about sub-staging T1 disease as T1a or T1b by EUS for ESCC. This study indicated that accuracy demonstrated by EUS for sub-staging T1a and T1b ESCC was only about 70%. Therefore, the technique of EUS requires further refinement to enhance its diagnostic accuracy for staging early ESCC. The results in this study indicated that EUS can stage and sub-stage early ESC pre-operatively whereas this modality need to be improved.

He LJ, Shan HB, Luo GY, Li Y, Zhang R, Gao XY, Wang GB, Lin SY, Xu GL, Li JJ. Endoscopic ultrasonography for staging of T1a and T1b esophageal squamous cell carcinoma. *World J Gastroenterol* 2014; 20(5): 1340-1347 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1340.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1340

INTRODUCTION

In accordance with the American Joint Committee on Cancer (AJCC) TNM classification of carcinoma of the esophagus and the esophageal-gastric junction (7th edition, 2010), the T1 stage of esophageal squamous carcinoma (ESCC) is subdivided into T1a (the tumor has infiltrated into the mucosal lamina propria and muscularis mucosa) and T1b (the tumor has infiltrated into the submucosa)[1,2]. Lymph node metastases occur in approximately 3%-6% of T1a cases, and T1a cancers can be removed surgically or with endoscopic treatments such as endoscopic mucosal resection (EMR) or endoscopic submucosal dissection, both of which have advantages of less damage and lower cost than esophagectomy[3-5]. In contrast, lymph node metastases occur in 21%-24% of those with T1b stage of ESCC, and these patients are not suitable for endoscopic treatment and require surgical resection[4-8]. Therefore, identifying the lesion stage of ESCC prior to treatment is essential.

The T staging classification depends mainly on the depth of the infiltration and the extent of damage to histological layers. At present, the most effective method for diagnosing and defining ESCC is endoscopic ultrasonography (EUS)[9,10]. EUS delivers the ultrasound probe via endoscopy and directly observes echoes in different layers of the digestive tract wall, and therefore, the depth of lesion infiltration and the extent of damage in different layers can be relatively easily visualized using this approach. In contrast, methods such as computed tomography (CT), magnetic resonance imaging (MRI) and positron emission computed tomography (PET-CT) cannot provide an accurate assessment of the different layers of the esophageal wall. In addition, the accuracy of EUS for T1 staging in ESCC is greater than that of other methods[11-13]. A meta-analysis demonstrated that the sensitivities of EUS in stages T1, T2, T3 and T4 are 81.6%, 81.4%, 91.4% and 92.4%, with corresponding specificities of 99.4%, 96.3%, 94.4% and 97.4%, respectively. However, there are few reports that have distinguished between T1a and T1b stages of ESCC using EUS[14]. In addition, this meta-analysis enrolled most pathological types of esophageal carcinoma, including adenocarcinoma, adenosquamous carcinoma and sarcoma, but there was a paucity of data concerning ESCC[15-17]. Due to the importance of preoperative staging in patients with stage T1a or T1b ESCC, a statistical analysis of the accuracy of EUS for identifying specific stages is needed[18,19]. This study focused on the role of EUS in distinguishing between the T1a and T1b stages of ESCC, to facilitate accurate preoperative assessment and more reliable decision-making on the appropriateness of endoscopic treatment or surgical resection.

MATERIALS AND METHODS

Patients

From January 2005 to December 2011, 72 ESCC patients who underwent endoscopic treatment or surgical resection and whose cancers were confirmed as T1N0M0 post-operatively were enrolled. Then, we retrospectively compared the preoperative EUS stages with the pathological results. The study protocol was approved by the institutional review board. All patients provided written informed consent before preoperative EUS examination.

In addition, to compare the accuracy of EUS for staging early and advanced ESCC, 90 randomly chosen patients with non-T1 ESCC (30 cases each of T2, T3 and T4, all confirmed post-operatively) were also enrolled.

Device

An Olympus GF-UM2000 EndoEcho Ultrasonic Gastrovideoscope was used for the study, and the EUS transducer offered four frequency modes, 5.0, 7.5, 12.0 and 20 MHz, to suit the clear-view requirement. Esophageal lesions were observed using the balloon method and the sterile deaerated water immersion method. All enrolled cases were observed using EUS with 7.5 and 12 MHz probes. The EUS examinations were performed by two experts in endoscopy, both of whom had at least 10 years of experience in Sun Yat-sen University Cancer Center. Both the balloon method and the water immersion method were performed during EUS examination.

EUS staging standard

Lightdale *et al*[20] stated that the esophageal wall can be divided into five layers by ultrasound. The first layer is a high echogenic band, which represents the interface echo of the mucosa; the second layer is a low echogenic band, which represents the muscularis mucosae; the third layer is a high echogenic band, which represents the submucosa; the fourth layer is a low echogenic band, which represents the muscularis propria; and the fifth layer is a high echogenic band, which represents the serosal layer, or the adventitia and the interface. Therefore, with EUS, ultrasonic images of stage T1a ESCC can identify significant thickening of the first and/or second layers, as well as an intact third layer. In contrast, the imaging of stage T1b ESCC shows continuous infiltration from the first to the third layer; the third layer becomes narrower, and the fourth layer remains complete or intact. Two experts in endoscopy conducted staging for all patients independently, thus creating two sets of staging data. When the results of both sets were consistent, the data were used in the analysis. When the results were not consistent, the experts discussed and read the images together to obtain a third common set of results.

Statistical analysis

Data from the two independent assessors were statisti-

Table 1 Characteristics of the 72 patients with early esophageal squamous cell carcinoma of T1a or T1b stage *n* (%)

Characteristics	Data
Age (yr)	
≤ 58[1]	36 (50)
> 58	36 (50)
Sex	
M	47 (65.3)
F	25 (34.7)
T1 stage	
T1a	35 (48.61)
T1b	37 (51.39)
Differentiation	
Carcinoma *in situ*	26 (36.11)
Well	6 (8.33)
Moderately	30 (41.67)
Poorly	10 (13.89)
Site of neoplasm	
Upper third	9 (12.50)
Mid third	52 (72.22)
Lower third[2]	11 (15.28)

[1]Median age; [2]The lower third not including the squamous cell carcinoma in the esophageal-gastric junction. M: Male; F: Female.

cally compared using SPSS version 13.0 software (SPSS Inc., Chicago, IL).

Numerical data are expressed as frequencies and percentages. A statistical descriptive method was used to analyze EUS evaluation indices for the clinical value of preoperative T and N staging of ESC, and this analysis included the diagnostic accuracy (AC), sensitivity (SE), false positive rate (FPR), false negative rate (FNR), specificity (SP), positive predictive value (PPV) and negative predictive value (NPV). The χ^2 test was used to analyze interclass differences in numerical data between the EUS and pathological results.

RESULTS

Clinical pathological characteristics of 72 patients with stage T1 ESCC

There were 47 males (65.3%) and 25 females (34.7%) with T1 ESCC. Their ages varied from 37 to 78 years, with a median of 58 years. Pathological examination revealed that 35 patients had stage T1a (48.61%) disease and 37 patients had stage T1b (51.39%) disease. Pathologically, there were 26 cases of carcinoma in situ (36.11%), six cases of well-differentiated squamous cell carcinoma (8.33%), 30 cases of moderately differentiated (41.67%), and 10 cases of poorly differentiated (13.89%). Regarding lesion location, 9 patients had lesions in the upper esophagus (12.50%), 52 had lesions in the mid-esophagus (72.22%), and 11 had lesions in the lower esophagus (15.28%) (Table 1).

Diagnostic value of EUS for stage T1 ESCC

Based on EUS results, the first expert concluded that 36 cases were stage T1a and 36 cases were stage T1b (Figure 1). Pathological results confirmed an AC of 65.3% (47/72), SE of 65.7%, FPR of 35.1%, FNR of 34.3%, SP of 64.9%, PPV of 63.9% and NPV of 66.7%. The area under the curve (AUC) was 0.668 (P = 0.014). The second expert concluded that 39 cases were stage T1a and 33 were T1b, with an AC of 66.7% (48/72), SE of 64.1%, FPR of 35.9%, FNR of 30.3%, SP of 69.7%, PPV of 71.4%, NPV of 62.2% and an AUC of 0.668 (P = 0.014). However, the combined assessment concluded that 36 cases were stage T1a and 36 were stage T1b. Pathological results confirmed an AC of 70.8% (51/72), SE of 74.3%, FPR of 27.0%, FNR of 25.7%, SP of 73.0%, PPV of 72.2%, NPV of 75.0% and AUC of 0.736 (P = 0.001, Table 2). These results indicate that EUS is a valid method for diagnosing and identifying stages T1a and T1b disease.

Impact of lesion location on EUS diagnosis of stage T1 ESCC

We conducted statistical analyses of the impact of tumor site on EUS staging accuracy for T1 stage ESCC. The combined set of common results revealed that for ESC in the upper thoracic region, EUS had an AC of 77.8% (7/9), SE of 80.0%, SP of 75.0%, PPV of 80.0% and NPV of 77.8%. For ESCC in the middle thoracic region, EUS had an AC of 73.1% (38/52), SE of 68.0%, SP of 77.8%, PPV of 73.9% and NPV of 72.4%. Finally, for ESC in the lower thoracic region, EUS had an AC of 72.7% (8/11), SE of 83.3%, SP of 60.0%, PPV of 71.4% and NPV of 75.0%. Furthermore, when compared with the combined set of common results, data obtained by the experts individually were not significantly better (Table 3).

Analysis of clinicopathological features related to the accuracy of EUS for distinguishing T1a or T1b ESCC

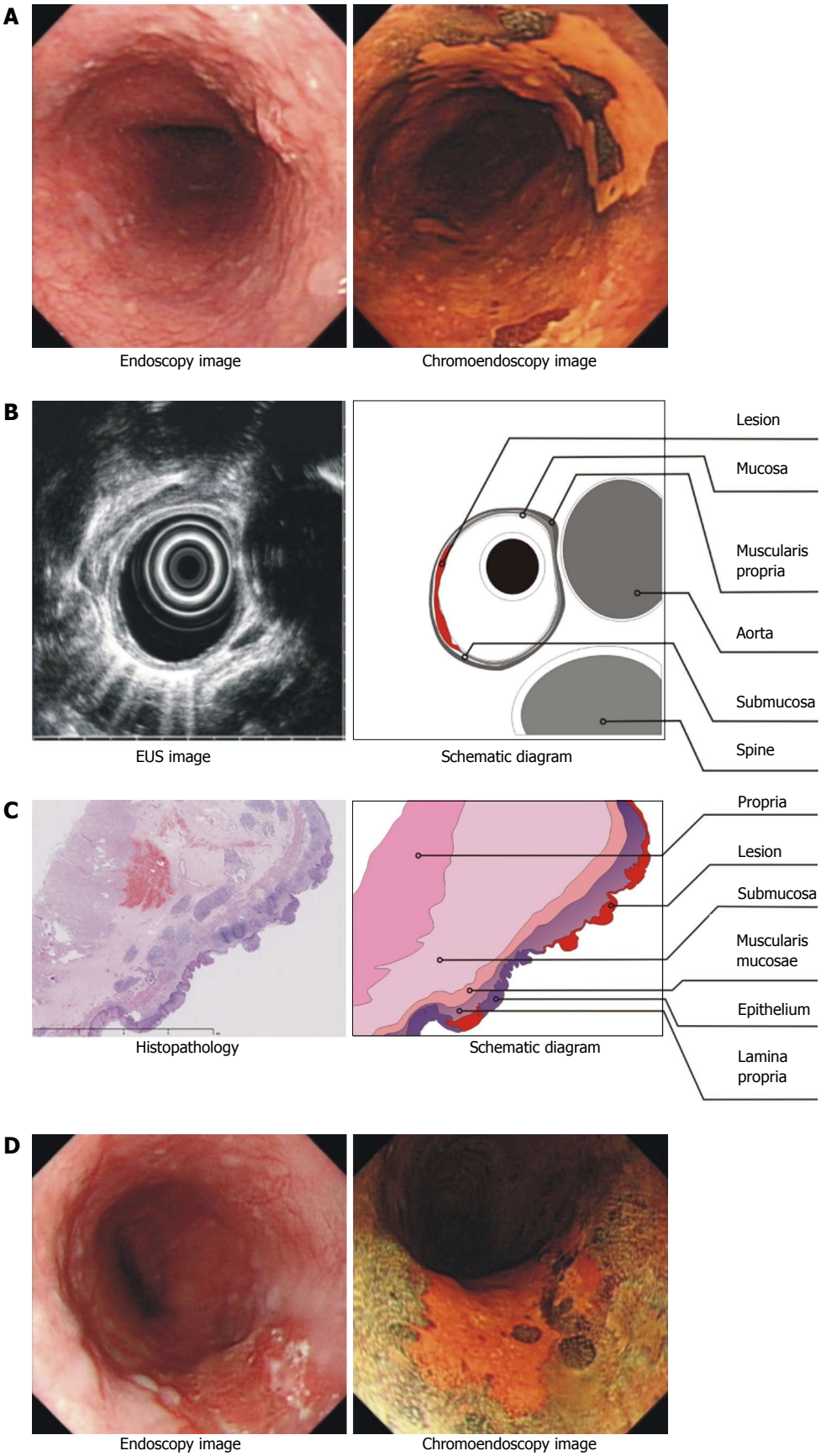
A multivariate statistical analysis of the accuracy of the distinguishing set was conducted (Table 4). The accuracy was closely related to tumor length (F = 4.984, P = 0.029) but not to tumor width (all P values > 0.05).

Comparison of the accuracy of EUS for distinguishing T1 and non-T1 ESCC

Given that the images of those with stage T1 and those with non-stage T1 disease were randomly arranged, the experts evaluated the images together. The combined set of common results had an AC of 93.21% (151/162), PPV of 92.5% and NPV of 92.8%. Among the 11 cases that were misdiagnosed, 7 cases with stage T1 were misdiagnosed and were re-staged based on the pathological results: 2 cases were T1a and 5 cases were T1b disease. Four patients in the non-stage T1 group were misdiagnosed as stage T1, and the pathological results showed that all 4 cases were stage T2 disease. These results demonstrate that EUS has a high accuracy and reliability for distinguishing stage T1 ESCC from non-T1 ESCC.

DISCUSSION

Early diagnosis and treatment are keys to reducing the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Epithelium
Lamina propria
D
Endoscopy image
Chromoendoscopy image

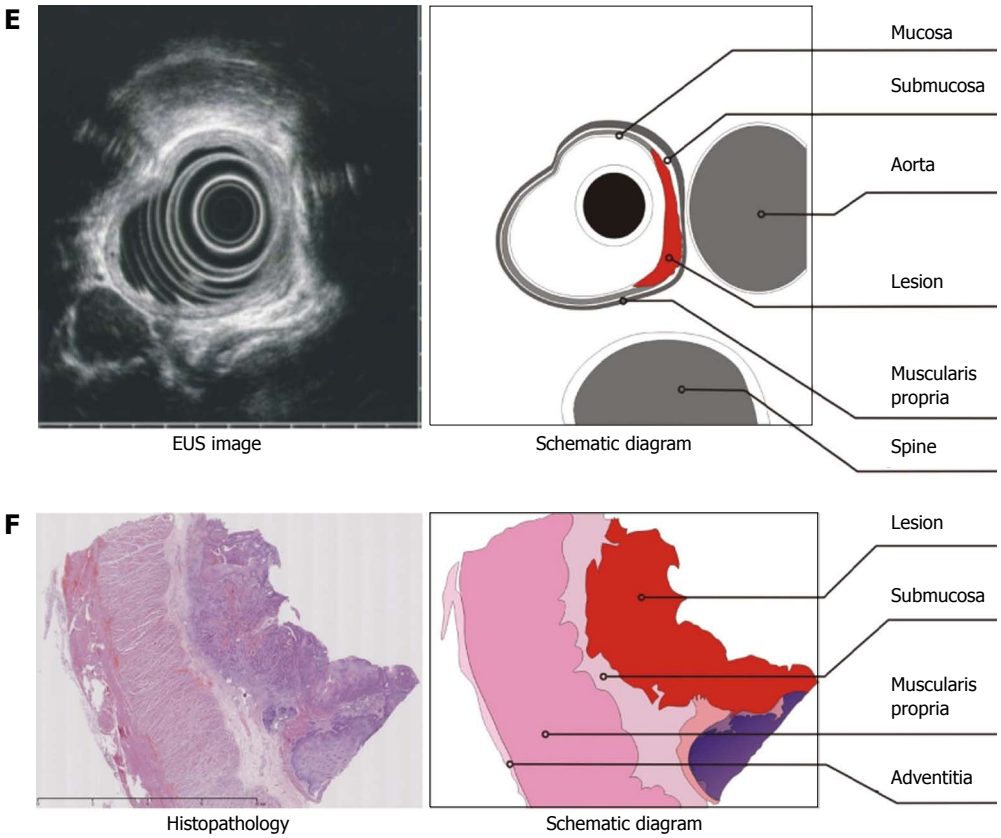

Figure 1 Images of patients with stage T1a or T1b early esophageal squamous carcinoma. A-C: Stage T1a early esophageal squamous carcinoma; A: The lesion visualized by endoscopy and chromoendoscopy with iodine staining; B: The lesion visualized by ultrasonography. The lesion appeared as a hypoechoic line in the first layer, and the outside edge of the second layer was smooth; these two layers represent the mucosa and muscularis mucosa, and thus, the lesion was limited to the mucosa. The third layer (hyperechoic), which represented the submucosa, was intact; C: The post-operative pathology revealed that the tumor had invaded the lamina propria; therefore, this case was confirmed as T1a stage; D-F: Stage T1b early esophageal squamous carcinoma; D: The lesion visualized by endoscopy and chromoendoscopy with iodine staining; E: The lesion visualized by ultrasonography appeared as a hypoechoic line not only in the first and second layers but also in the third layer (hyperechoic), which represents the submucosa; the fourth layer (hypoechoic), which represents the muscularis propria, was intact; F: The postoperative pathology confirmed that the lesion covered the mucosa and had invaded into the submucosa; therefore, the patient was staged as T1b.

morbidity and mortality associated with ESCC. With recent developments in endoscopic techniques, endoscopic treatment has become a standard first-line management strategy for early ESCC and precancerous lesions. However, due to significant differences in the presence of lymph node metastases and the prognosis of patients with stage T1a ESCC compared to those with stage T1b ESCC, accurate staging is necessary to choose the most appropriate treatment. The 2011 NCCN Guidelines state that stage T1a esophageal cancer (EC) is an indication for endoscopic treatment. Because imaging methods such as CT, MRI, and PET-CT cannot distinguish the layers of the esophageal wall, they are unable to accurately distinguish between T1a and T1b. Preoperative EUS can provide accurate information on echoes, the depth of lesion infiltration, and even the interface between the esophageal wall layers. Therefore, EUS is widely used clinically so that the most appropriate treatment can be chosen[21].

Pech *et al*[22] demonstrated an EUS accuracy of 92% for diagnosing stage T1 EC, which was higher than the reported accuracy for stages T3 and T4. This is essentially consistent with our results (93.21%). This observation may be explained by the fact that in stage T1, the extent of tumor infiltration ranges from the first layer to the third layer, and these three layers, particularly the submucosa, are relatively thick and significantly different from the muscular layer in terms of echo grayscale. Therefore, EUS has a high accuracy for evaluating early tumor infiltration. Hence, we believe that EUS is reliable for staging T1 ESCC. However, few studies have focused on the accuracy of EUS for sub-staging T1 esophageal carcinoma. There has been only one relevant study[17], which reported an accuracy of 86% (19/22) for stage T1a and an accuracy of 60% (22/33) for stage T1b. However, of the 55 cases in that study, 33 were adenocarcinomas, of which, 27 had developed from Barrett's esophagus, and most of the EUS images showed thickening of the muscularis mucosae rather than the mucosal layer. Our study, on the other hand, focused on ESCC, which is a tumor that has a high occurrence rate in South China, generally develops in the mucosal layer and gradually infiltrates into the submucosa. Therefore, reliable statistical data about the accuracy of EUS for diagnosing ESCC stages T1a and T1b are needed, and some possible clinical and pathological factors that may impact the diagnostic accuracy of EUS should be considered.

Table 2 Accuracy of endoscopic ultrasound for staging T1a or T1b esophageal squamous cell carcinoma

Pathology	Expert 1		Expert 2		Common results
	T1a (n = 36)	T1b (n = 36)	T1a (n = 39)	T1b (n = 33)	T1a (n = 36)
T1a (n = 35)	23	13	25	10	26
T1b (n = 37)	13	24	14	23	10
Group					
AC	65.30%		66.70%		70.80%
SE	65.70%		64.10%		74.30%
FPR	35.10%		35.90%		27.00%
FNR	34.30%		30.30%		25.70%
SP	64.90%		69.70%		73.00%
PPV	63.90%		71.40%		72.20%
NPV	66.70%		62.20%		75.00%
AUC	0.653		0.668		0.736
P value	0.026		0.014		0.001

AC: Accuracy; SE: Sensitivity; FPR: False positive rate; FNR: False negative rate; SP specificity; PPV: Positive predictive value; NPV: Negative predictive value. AUC: Area under the curve.

Table 3 Accuracy of endoscopic ultrasound for staging T1a or T1b esophageal squamous cell carcinoma in relation to tumor location

Site of neoplasm		Expert 1		Expert 2		Common results	
		T1a	T1b	T1a	T1b	T1a	T1b
Upper third	T1a	4	2	4	2	4	1
	T1b	1	2	1	2	1	3
Mid third	T1a	14	10	16	11	17	8
	T1b	9	19	7	18	6	21
Lower third	T1a	5	1	5	1	5	1
	T1b	2	3	2	3	2	3
Group							
AC	Upper third	66.7%		66.7%		77.8%	
	Mid third	63.50%		65.40%		73.10%	
	Lower third	72.70%		72.70%		72.70%	
SE	Upper third	66.70%		66.70%		80.00%	
	Mid third	58.30%		59.30%		68.00%	
	Lower third	83.30%		83.30%		83.30%	
FPR	Upper third	66.70%		66.70%		75.00%	
	Mid third	67.90%		72.00%		77.80%	
	Lower third	60.00%		60.00%		60.00%	
FNR	Upper third	80.00%		80.00%		80.00%	
	Mid third	60.90%		69.60%		73.90%	
	Lower third	71.40%		71.40%		71.40%	
SP	Upper third	50.00%		50.00%		75.30%	
	Mid third	65.50%		62.10%		72.40%	
	Lower third	75.00%		75.00%		75.00%	

AC: Accuracy; SE: Sensitivity; FPR: False positive rate; FNR: False negative rate; SP: Specificity.

Because relatively few patients with ESCC have stage T1N0M0 disease, our retrospective study enrolled only 72 cases from 2005 to 2011. Both endoscopists had 10 years of experience in endoscopic diagnosis, and their individual assessments revealed similar figures in terms of accuracy of diagnosis (65.3% *vs* 66.7%). In addition, data on accuracy from the combined assessment did not reveal a significant increase (70.8%, $P > 0.05$). Low diagnostic accuracy might be explained by a few reasons[23]. First, the esophageal wall is thin, and it is difficult to distinguish the boundaries of various layers according to the gray scale in EUS images alone. Second, the boundaries in EUS images are actually the reflections of the interfaces between various layers, and there is little difference between the actual anatomical structure and the echoic gray scale of EUS. Third, the two experts made the diagnosis through static images, without consistent and dynamic ultrasonic images. Moreover, this study focused on early ESCC, yet previous reports included all pathological types, such as squamous carcinoma, adenocarcinoma, adenosquamous carcinoma and sarcoma. Additionally, in most early ESCC patients, inflammation was present around the lesions, especially in the lower esophagus (*e.g.*, the impact of reflux esophagitis), resulting in changes in the layers of the esophageal wall (particularly when shown as thickening of the esophageal submucosa), which may have an im-

Table 4 Cox multivariate analyses of factors affecting diagnostic accuracy of endoscopic ultrasound for staging T1a or T1b esophageal squamous cell carcinoma

Characteristics	Hazards ratio	*P* value
Age (≤ 58 *vs* > 58 yr)	1.240	0.269
Sex (male *vs* female)	0.923	0.340
Tumor width, cm (≤ 2 *vs* > 2)	1.407	0.240
Tumor length, cm (≤ 2 *vs* > 2)	4.984	0.029[1]
Differentiation (well-moderate *vs* poor)	2.815	0.098

[1]A probability value of less than 0.05 was considered significant.

pact on diagnosis.

In addition, we found that the accuracy of EUS for staging T1 was related to tumor site and length. The diagnostic accuracy of both experts was lower for lesions in the middle section than in the upper and lower sections. The common results revealed that the diagnostic accuracy of lesions in the middle section increased to 73.1%, which was slightly higher than the accuracy observed for the lower section (72.7%), but still lower than the accuracy of for the upper section (77.8%). We believe that this difference may be explained by several factors. First, a minority of ESCC cases originated in the upper and lower sections (9 and 11, respectively), which may have adversely affected the statistical accuracy. Second, the data also showed that T staging of ESCC in the middle section was more difficult, and both experts had lower accuracy rates in this area (63.5% and 65.4%), which necessitated negotiation or discussion (which generated the common results) that increased the accuracy rate (73.1%). Third, because the esophagus is relatively wide in its thoracic section, it is difficult to fill the lumen with water during EUS examination; therefore, it is hard for the ultrasonic probe to get close to the lesion. Finally, the middle section of the esophagus is near the left atrium of the heart and the thoracic aorta[24,25], both of which pulsate and make it difficult to image. Consequently, T1 staging of ESCC in the middle section is more difficult. Furthermore, our multivariate statistical analysis demonstrated that accuracy was related to tumor length (maximum vertical axis of parallel esophagus) ($P = 0.029$), but not to tumor width (maximum vertical axis of vertical esophagus) or the degree of differentiation ($P = 0.098$). This result may be explained by the fact that when the tumor is long, the ultrasonic probe has to be moved backwards and forwards to visualize the lesion. At present, circle-scan probes are used in most clinical situations, which can comprehensively scan tumor width, and thus, width does not necessarily affect diagnostic accuracy.

Our team had tried to employ submucosal saline injection (a novel technique) to improve EUS for staging and sub-staging early ESCC. The preliminary results revealed that the accuracy of SSI + EUS for staging or substaging early ESCC increased significantly[26,27].

In conclusion, EUS plays an important role in defining the T stage of ESCC. However, accurately staging early ESCC as T1a or T1b is difficult using EUS alone, especially when the lesion is located in the mid-thoracic section. Therefore, EUS as a technique needs to be improved to distinguish T1a and T1b disease in patients with early ESCC.

COMMENTS

Background

Correct staging and sub-staging of early esophageal squamous cell carcinoma (ESCC) is of great importance for guiding therapy. Endoscopic ultrasound (EUS) is the most common modality for staging early ESCC.

Research frontiers

However, the accuracy of EUS for staging ESCC is not good and diverse. In addition, there is no other report about sub-staging T1 disease as T1a or T1b by EUS for ESCC.

Innovations and breakthroughs

This study investigated whether EUS can distinguish between T1a and T1b ESCC. The results indicated that accuracy demonstrated by EUS for sub-staging T1a and T1b ESCC was only about 70%. Therefore, the technique of EUS requires further refinement to enhance its diagnostic accuracy for staging early ESCC.

Applications

The results in this study revealed that EUS can stage and sub-stage early ESCC preoperatively whereas EUS should be improved to better stage early ESCC.

Peer review

This is a retrospective study for diagnostic value of EUS for early esophageal cancer with squamous cell carcinoma. The authors concluded that EUS demonstrated median degree of accuracy for distinguishing between T1a and T1b ESCC, and so it is necessary to improve EUS for staging and sub-stage early ESCC.

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BRIEF ARTICLE

Oleanolic acid and ursolic acid inhibit proliferation in transformed rat hepatic oval cells

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Abstract

AIM: To investigate H_2O_2-induced promotion proliferation and malignant transformation in WB-F344 cells and anti-tumor effects of ursolic acid (UA) and oleanolic acid (OA).

METHODS: WB-F344 cells were continuously exposed to 7 x 10^{-7} mol/L H_2O_2 for 21 d. Observations of cell morphology, colony formation rates, flow cytometric analysis of cell cycle changes and aneuploidy formation indicated that H_2O_2 was able to induce malignant transformation of WB-F344 cells. We treated malignantly transformed WB-F344 cells with 4 μmol/L OA or 8 μmol/L UA for 72 h and analyzed the cell cycle distribution by flow cytometry.

RESULTS: MTT assay showed that 7 x 10^{-7} mol/L H_2O_2 decreased G_1 phase subpopulation from 73.8% to 49.6% compared with the control group, and increased S phase subpopulation from 14.5% to 31.8% ($P < 0.05$ *vs* control group). Cell morphology showed that nucleus to cytoplasm ratio increased, many mitotic cells, prokaryotes and even tumor giant cells were shown in H_2O_2-induced WB-F344 cells. Fluorescence activated cell sorting analysis showed that WB-F344 cell aneuploidy increased to 12% following H_2O_2 treatment. Flow cytometric analysis of the transformed WB-F344 cells following treatment with OA (4 μmol/L) and UA (8 μmol/L) showed that OA increased G_1 subpopulation to 68.6%, compared to 49.7% in unexposed cells. UA increased G_1 subpopulation to 67.4% compared to 49.7% in unexposed cells ($P < 0.05$ *vs* H_2O_2 model group).

CONCLUSION: H_2O_2 causes the malignant transformation of WB-F344 cells. OA and UA exert anti-tumor effects by inhibiting the proliferation in malignantly transformed WB-F344 cells.

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Key words: Oxidative stress; Hepatocarcinogenesis; Malignant transformation; Oleanolic acid; Ursolic acid

Core tip: It is known that H_2O_2 can promote tumorigenesis. Here, we used H_2O_2 as a premalignant and malignant agent to induce proliferation and malignant transformation in quiescent rat liver oval cell line WB-F344. Multistage carcinogenic processes provide the basis for interrupting and reversing precancerous changes; therefore, reversing precancerous changes is critical for tumor prevention and treatment. The salient and novel findings of the study are that ursolic acid (UA) and oleanolic acid (OA) induced malignantly transformed WB-F344 cell arrest in the G_1 phase and apparently

inhibited the proliferation of these cells. These results better our understanding of the antitumor effects of OA and UA.

Han YY, Xue XW, Shi ZM, Wang PY, Wu XR, Wang XJ. Oleanolic acid and ursolic acid inhibit proliferation in transformed rat hepatic oval cells. *World J Gastroenterol* 2014; 20(5): 1348-1356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1348.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1348

INTRODUCTION

Hepatocarcinogenesis is a multistage developmental process that is divided into three stages: initiation, promotion and progression[1]. The initiation stage is characterized by irreversible genetic mutations. The promotion stage is characterized by a process of clonal cell hyperplasia, a process that is generally considered to be reversible, thus being an ideal target for cancer prevention[2]. The promotion stage is also referred to as the stage of hepatic precancerous lesions, and its principle pathological feature is oval cell hyperplasia. Oval cells are involved in liver regeneration and can mobilize and differentiate into hepatocytes and bile duct cells when the liver is injured. However, changes in the liver microenvironment can lead to the malignant transformation of oval cells during the process of hyperplasia, which is involved in the initiation and promotion stages of liver cancer development[3-5].

Reactive oxygen species (ROS) induce oxidative stress, serve as a major intrinsic factor in multistage carcinogenic processes and are involved in every step of this progression. Therefore, ROS and their downstream effects may be targets for cancer prevention[6,7]. Our previous studies demonstrated that ROS levels were significantly increased in aflatoxin- and diethylnitrosamine-induced rat hepatic precancerous lesions, whereas the activity of antioxidant enzymes decreased significantly[8]. To further explore the mechanism of ROS-induced hepatocellular carcinoma (HCC) development, we used 7×10^{-7} mol/L hydrogen peroxide (H_2O_2) as a premalignant and malignant agent to induce proliferation and malignant transformation in quiescent rat hepatic oval cells (WB-F344 cells). In this way, a model of oval cell malignant transformation was established[9]. Multistage carcinogenic processes provide the basis for interrupting and reversing precancerous changes. Ursolic acid (UA) and its isomer oleanolic acid (OA) are triterpenoic acids that naturally coexist in many Chinese herbs, such as cornel[10], glossy privet fruit[11] and Chinese hawthorn[12]. On the basis of our previous studies, we here explored the efficacy of OA and UA in the prevention and treatment of precancerous changes. Flow cytometry analysis of cell cycle distribution revealed differences in malignantly transformed WB-F344 cells following UA and OA treatment. We observed that OA and UA can inhibit the proliferation of malignantly transformed WB-F344 cells, which may explain the anti-tumor effects of UA and OA.

MATERIALS AND METHODS

Cell culture

The rat oval cell line WB-F344 (a gift from Dr. Geng-Tao Liu, Peking Union Medical College) was grown in high-glucose Dulbecco's Modified Eagle Medium (DMEM) with 100 U/mL penicillin and 100 μg/mL streptomycin and with or without 10% fetal bovine serum (FBS). The cells were maintained in the logarithmic growth phase at 37 ℃ in 5% CO_2. The buffalo rat liver cells (BRL) rat hepatocyte cell line (purchased from Life Research Institute, Chinese Academy of Sciences, Shanghai, China) was cultured in high-glucose DMEM media that was supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. These cells were maintained in the logarithmic growth phase at 37 ℃ in 5% CO_2. The WB-F344 cells were stimulated with 7×10^{-7} mol/L H_2O_2 for 12 h per day. After 21 d, these cells served as the malignant transformation model group cells (H_2O_2). The WB-F344 cells that were cultured in complete medium were used as one control group. The cells in both of these groups were cultured for Western blotting and flow cytometric analyses.

Drugs and drug treatment

OA, UA and fluorouracil (5-FU) were purchased from Sigma (St Louis, MO, United States). OA and UA were dissolved in dimethyl sulfoxide (DMSO) and added to the cultures at a final concentration of 4 μmol/L or 8 μmol/L for 24, 48, 72 or 96 h. For the control experiments, an equal volume of DMSO was added to the control group. Fluorouracil (5-FU), a positive control, was added to the cultures at a final concentration of 0.05 mmol/L for 24, 48, 72 or 96 h.

Colony forming cell assays

The model group and the control group cells were resuspended in DMEM that was mixed with methylcellulose, which consisted of 1% methylcellulose, 10% bovine serum albumin, 100 U/mL penicillin and 100 μg/mL streptomycin. Next, 500 cells were suspended in 1.0 mL and were then plated in 24-well plates and cultured for 7-10 d. The colonies were counted at 400 × magnification under microscope.

Cell proliferation assay (MTT method)

This assay was performed as described previously[13], with slight modifications. Briefly, 24 h prior to the experiment, the WB-F344 cells and the transformed WB-F344 cells were added into 96-well dishes. The WB-F344 cells were exposed to 7×10^{-4}-7×10^{-9} mol/L H_2O_2 for 6, 9, 12, 15, 18 h. The transformed WB-F344 cells were treated with OA at 0.5, 1, 2, 4 or 8 μmol/L OR WITH UA at 1, 2, 4, 8 or 16 μmol/L and allowed to incubate for 24, 48, 72 or 96 h. Prior to harvesting, 20 μL of 5 mg/mL

MTT [3-(4,5-dimethythiazolzyl)-2,5-diphenyl tetrazolium bromide; Sigma, St Louis, MO, United States] was added to each well. After incubating for 4 h, 0.2 mL of DMSO was added to stop the reactions. The absorbance values for each well were determined spectrophotometrically at 490 nm on a Microplate Reader (BIO-TEK, Rockville, MA, United States).

Flow cytometry (FACS)

The cell cycle analysis was performed as previously described[14]. Briefly, aliquots of cells (1.5×10^6) were pelleted (1300 rpm × 5 min at 4 ℃) and were washed twice in ice-cold phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol overnight at 4 ℃, washed in PBS and digested with DNase-free RNase A (10 μg/mL) at 37 ℃ for 30 min. Prior to FACS analysis, the cells were resuspended in 200 μL propidium iodide (PI, 10 μg/mL; Sigma, St Louis, MO, United States) for DNA staining. A BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, United States) flow cytometer was used to analyze cellular DNA contents.

Statistical analysis

The experimental data were expressed as the mean ± SD, and the SPSS 11.5 statistical package was used for data analysis. The data were compared using single factor analysis of variance (one way). P values < 0.05 were considered statistically significant.

RESULTS

H_2O_2 promoted WB-F344 cell proliferation

To estimate the effects of H_2O_2 on cell proliferation, WB-F344 cells were exposed to 7×10^{-4}-7×10^{-9} mol/L H_2O_2 for 6, 9, 12, 15 and 18 h, respectively. Cell proliferation was evaluated using the MTT assay. Our results showed that 7×10^{-7} mol/L H_2O_2 promoted WB-F344 cell proliferation obviously (Figure 1A), so we used 7×10^{-7} mol/L H_2O_2 as a premalignant and malignant agent to induce proliferation and malignant transformation in quiescent rat hepatic oval cells. To determine whether the H_2O_2-induced effect on cell growth was closely related to cell cycle control, we determined the cell cycle distribution of WB-F344 cells using FACS analysis. In H_2O_2-exposed WB-F344 cells, the G1 phase subpopulation decreased from 73.8% to 49.6% compared with the control group, and the S phase subpopulation increased from 14.5% to 31.8% (Figure 1B and C). These results indicated that H_2O_2 promoted WB-F344 cell proliferation, an effect that is potentially involved in the carcinogenic effects of this ROS.

H_2O_2 induced WB-F344 malignant transformation

Cell morphology was observed under microscope to further investigate the H_2O_2-induced tumorigenicity of WB-F344 cells. The cells in the control group exhibited a regular shape and abundant cytoplasm and grew with contact inhibition. After H_2O_2 stimulation for 21 d, the cells became anomalous and changed in size. An increasing nucleus to cytoplasm ratio was observed (Figure 2A), as were many mitotic cells (Figure 2A), prokaryotes (Figure 2A) and even tumor giant cells (Figure 2A). Compared with normal WB-F344 cells, there was no contact inhibition between the cells, and overlapping growth was often present (Figure 2A). The cell morphologic changes indicated that H_2O_2 had induced the malignant transformation of WB-F344 cells. Moreover, H_2O_2-treated WB-F344 cells formed clones in methylcellulose medium culture (Figure 2B). These results indicate that oxidative stress plays an important role in the progression of hepatocarcinogenesis.

FACS analysis was used to examine the tumorigenicity of H_2O_2 in WB-F344 cells. DNA was stained with propidium iodide to analyze cellular DNA content. The population of > 4N cells represent aneuploidy cells. WB-F344 cell aneuploidy increased to 12% following H_2O_2 treatment (Figure 2C). Thus, H_2O_2 significantly induced aneuploidy in WB-F344 cells. These results suggest that H_2O_2 could cause the malignant transformation of WB-F344 cells.

OA and UA inhibited the proliferation of malignantly transformed WB-F344 cells but had no obvious effect in normal rat liver BRL cells

We analyzed the inhibitory effects of OA and UA on the proliferation of malignantly transformed WB-F344 cells using the MTT method. The time- and dose-effect curves revealed that 4 μmol/L OA or 8 μmol/L UA caused significant growth inhibition in malignantly transformed WB-F344 cells within 24-96 h (Figure 3A and B). Based on these results, we chose 4 μmol/L OA or 8 μmol/L UA as the final concentrations and 72 h as the drug intervention time. To estimate the genotoxicity of OA and UA, the normal rat liver cell BRL line was also exposed to 4 μmol/L OA or 8 μmol/L UA for 72 h. However, no obvious inhibitory effect on the growth of BRL cells was observed (Figure 3C). To estimate the genotoxicity of OA and UA on quiescent WB-344 cells, the quiescent WB-344 cells were also exposed to 4 μmol/L OA or 8 μmol/L UA for 72 h. Also no obvious inhibitory effect on the growth of quiescent WB-344 cells was observed (Figure 3D).

OA and UA induced the arrest of malignantly transformed WB-F344 cells in the G_1 phase

Using FACS analysis, we observed that treatment with 4 μmol/L OA increased the G_1 subpopulation of H_2O_2-treated WB-F344 cells to 68.6% within 72 h, compared to 49.7% in unexposed cells (Figure 4A and B). In H_2O_2-treated WB-F344 cells, 8 μmol/L UA increased the G1 subpopulation to 67.4% at 72 h, compared to 49.7% in unexposed cells. As a positive control, 5-FU also increased the G1 subpopulation to 75.5% compared to unexposed cells (49.7%). In addition, OA, UA and 5-FU decreased the size of the G_2/M phase and S phase subpopulations in transformed WB-F344 cells. These results

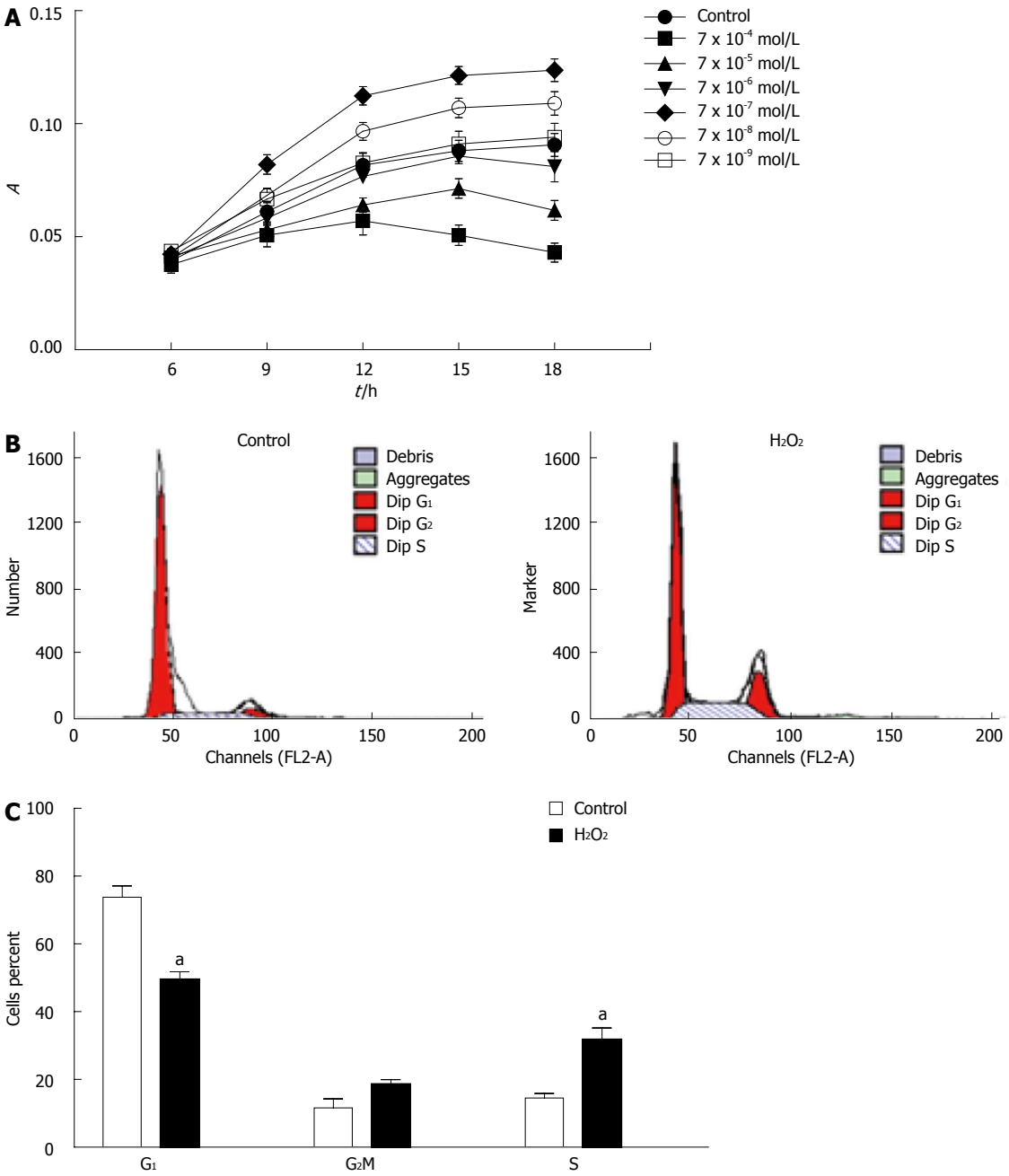

Figure 1 H_2O_2 promoted WB-F344 cell proliferation. A: The effect of H_2O_2 on cell proliferation. WB-F344 cells were exposed to 7×10^{-4}-7×10^{-9} mol/L H_2O_2 for 6, 9, 12, 15, and 18 h. Cell proliferation was evaluated using the MTT assay. The data represent the mean ± SD derived from three independent experiments; B: Flow cytometric analyses of the cell cycle in H_2O_2-treated WB-F344 cells. The cells in the control and the H_2O_2 groups were stained with 10 μg/mL propidium iodide, and the DNA content was analyzed as described in the Materials and Methods; C: Histograms indicating the percentages of WB-F344 cells in the control and H_2O_2 exposure groups in the G_1, G_2/M and S phases. The data represent the mean ± SD derived from three independent experiments. $^aP < 0.05$ *vs* control group.

indicate that OA and UA could inhibit DNA replication in malignantly transformed WB-F344 cells, leading to cell cycle arrest in the G_1 phase.

DISCUSSION

The hypothesis that cancers arise from stem cells is currently an active research field. The main premise of this hypothesis is that cancer results from a subset of cancer stem cells that do not mature into terminally differentiated cells but instead continue to proliferate[15]. Certain cancer cells can express the same surface markers as do stem cells, providing support for this theory[16,17]. Stem cell differentiation is affected by microenvironment changes in tissues and organs. When the stem cell microenvironment changes, it is possible for stem cells to malignantly differentiate, providing a certain degree of evidence that these cells are involved in tumorigenesis[18,19]. Many studies have demonstrated that oxidative stress produces excess ROS, which can be involved in various stages of tumor development[20]. ROS, as an important microenvironment factor, can play important roles in stem cell

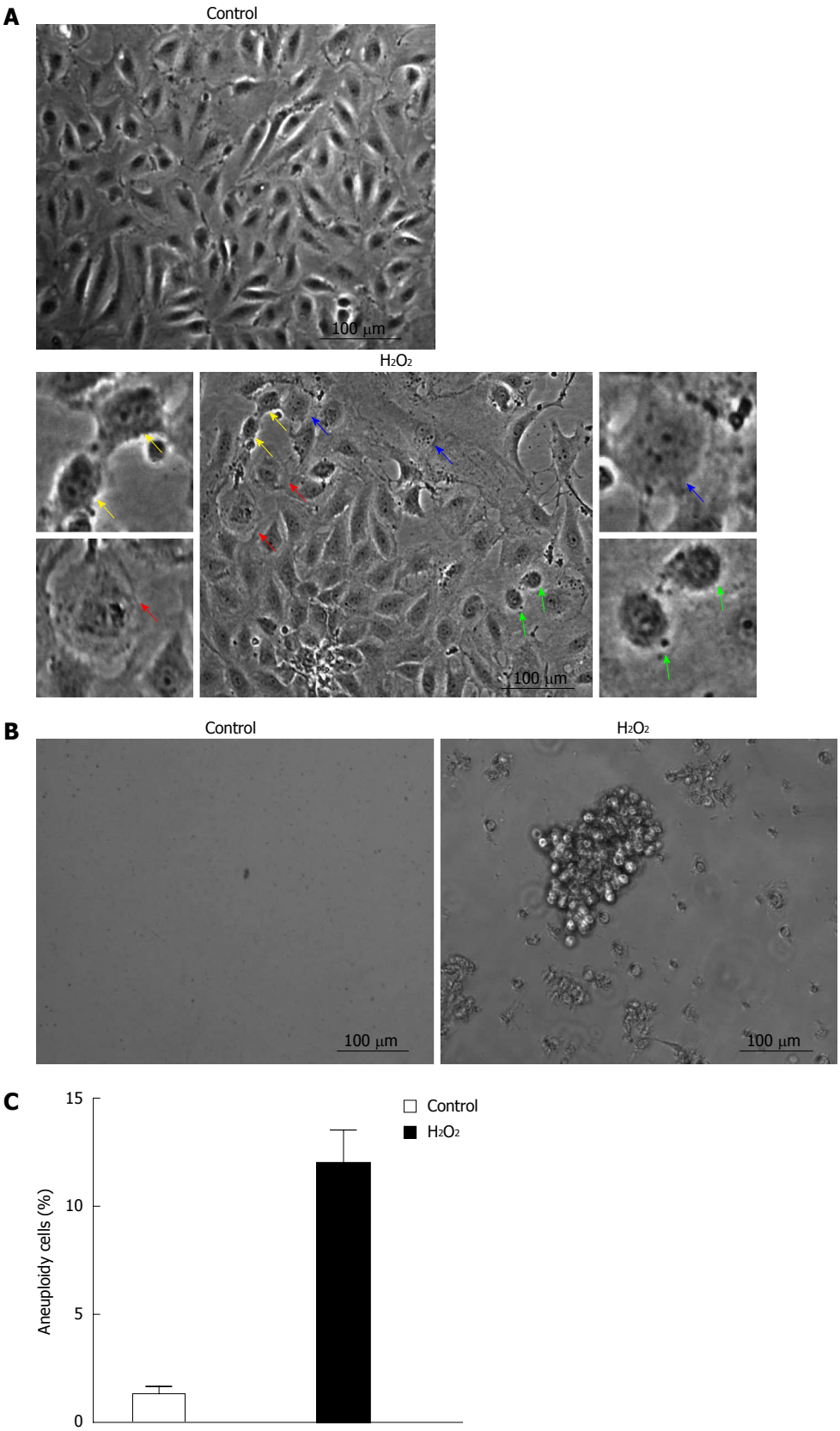

Figure 2 H_2O_2 induced WB-F344 malignant transformation. A: The morphology of H_2O_2-treated WB-F344 cells. The cells were cultured in complete medium (control) or treated with 7×10^{-7} mol/L H_2O_2 12 h per day for 21 d. Cell morphology was observed under microscope (× 400). An increasing nucleus to cytoplasm ratio was observed (green arrow), as were many mitotic cells (yellow arrow), prokaryotes (blue arrow) and even tumor giant cells (red arrow); B: The tumorigenicity of transformed WB-F344 cells as determined using the methylcellulose culture assay. Cell colony formation was observed under microscope (× 400); C: H_2O_2 increased WB-F344 cell aneuploidy. The cells were cultured in complete medium (control) or treated with 7×10^{-7} mol/L H_2O_2 12 h per day for 21 d and harvested for flow cytometry. DNA was stained with propidium iodide (10 μg/mL), and a BD FACS Calibur was used to analyze cellular DNA content. The population of > 4N cells represent aneuploidy cells. Histograms indicating the aneuploidy cells among the control cells and the H_2O_2-treated cells. The data represent the mean ± SD derived from three independent experiments.

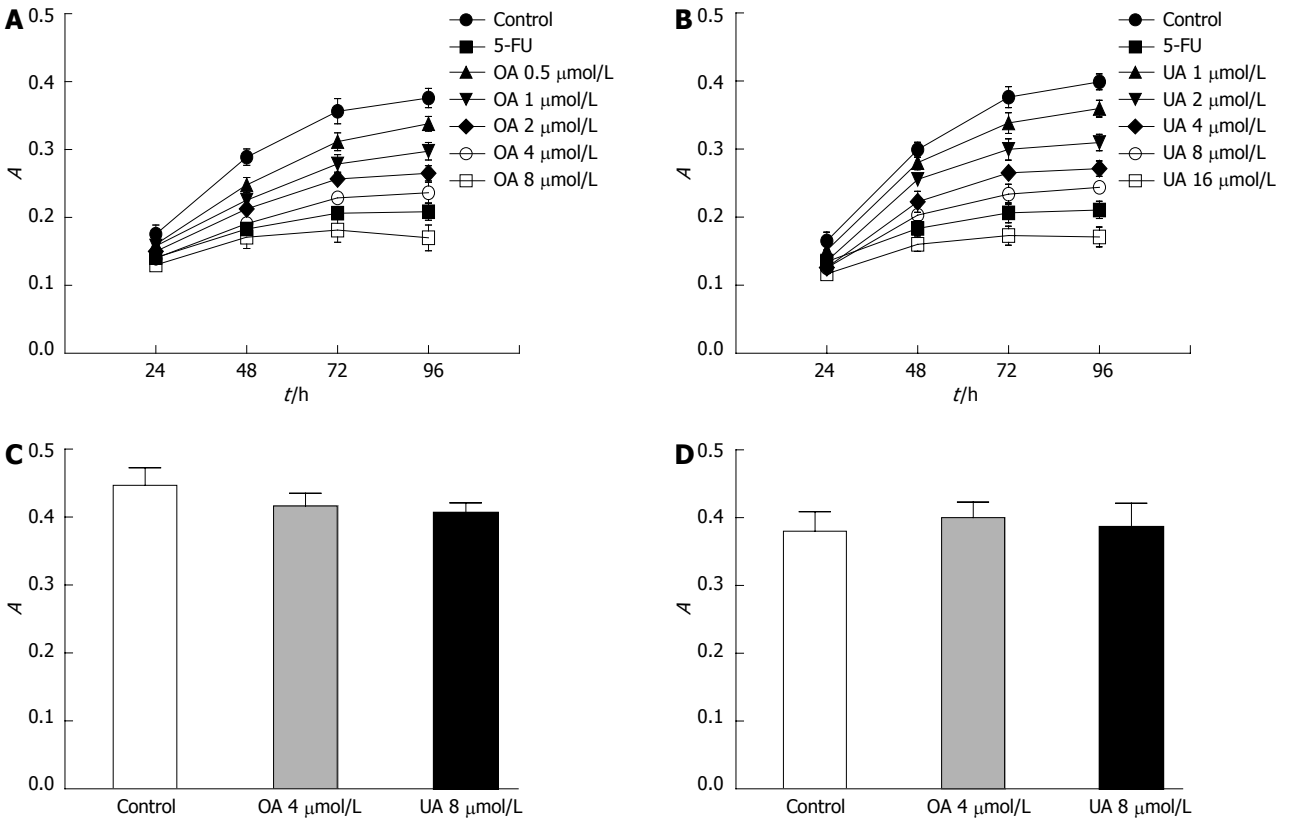

Figure 3 Effect of oleanolic acid and ursolic acid on cell proliferation. A, B: The inhibitory effect of oleanolic acid (OA) and ursolic acid (UA) on H_2O_2-induced cell proliferation. Cell proliferation was evaluated using the MTT assay (see Materials and Methods). The data represent the mean ± SD derived from three independent experiments; C: The effects of OA and UA on quiescent BRL rat liver epithelial cell proliferation. BRL cells were exposed to 4 μmol/L OA or 8 μmol/L UA for 72 h. Cell proliferation was evaluated using the MTT assay. The data represent the means ± SD derived from three independent experiments; D: The effects of OA and UA on quiescent WB-344 cells proliferation. WB-344 cells were exposed to 4 μmol/L OA or 8 μmol/L UA for 72 h. Cell proliferation was evaluated using the MTT assay. The data represent the mean ± SD derived from three independent experiments.

differentiation. Oval cells are hepatic stem cells with a distinct phenotype and have been demonstrated to be a bipotential progenitor of epithelial cells that are found in the liver, namely, hepatocytes and bile duct cells[21]. In the present study, we used a low dose of H_2O_2 to continuously stimulate WB-F344 cells (an *in vitro* model of liver oval cells) to explore the effects of oxidative stress on the malignant transformation of oval cells.

The MTT colorimetric experiments led us to select the concentration of 7×10^{-7} mol/L H_2O_2 to stimulate WB-F344 cells. This concentration, when applied to WB-F344 cells for 12 h per day for 21 d, clearly stimulated proliferation. Specifically, the treated cells exhibited characteristics of transformed cells, *e.g.*, mitotic activity, a loss of contact inhibition, and multinucleation, with tumor giant cells also being observed. The H_2O_2-treated cells were able to form colonies cultured in methylcellulose semi-solid medium, indicating that the anchorage dependence of cells was lost following H_2O_2 stimulation, which is an important feature of transformed cells[22]. It has been reported that cancer is a disease that is caused by a cell cycle disorder[23,24]. Mutations can be clonally amplified in the promotion, or precancerous lesion stage; therefore, cancer cells exhibit active proliferation and an increase in the S phase fraction[25]. Our results demonstrated that continuous stimulation of WB-F344 cells with H_2O_2 significantly increased the S phase fraction and the number of aneuploid cells. Increased aneuploidy is an important feature of tumor cells[26]. Flow cytometric analysis indicated that the proportion of hypotetraploid cells may have increased, but specific karyotype changes require further confirmation. The important features that were observed in the malignantly transformed cells provide strong evidence that the continuous stimulation of liver oval cells with H_2O_2 causes these cells to transform. Thus, a malignant transformation model was successfully created; however, the behavior of the transformed cells should be further confirmed by experiments in nude mice.

The prevention and treatment of liver cancer has been receiving a great deal of attention. Traditional Chinese medicines (TCMs), with their low toxicity and many targets, have huge advantages as potential treatments[27,28]. However, the pharmacological effects of TCM are complex. To further explore the mechanism of tumor suppression by TCM, the purification of the active ingredients and their use in research has become a popular area of research. UA and its isomer OA are triterpenoic acids that coexist in many Chinese herbs. There are multiple uses of TCM herbs that contain UA and OA. To further

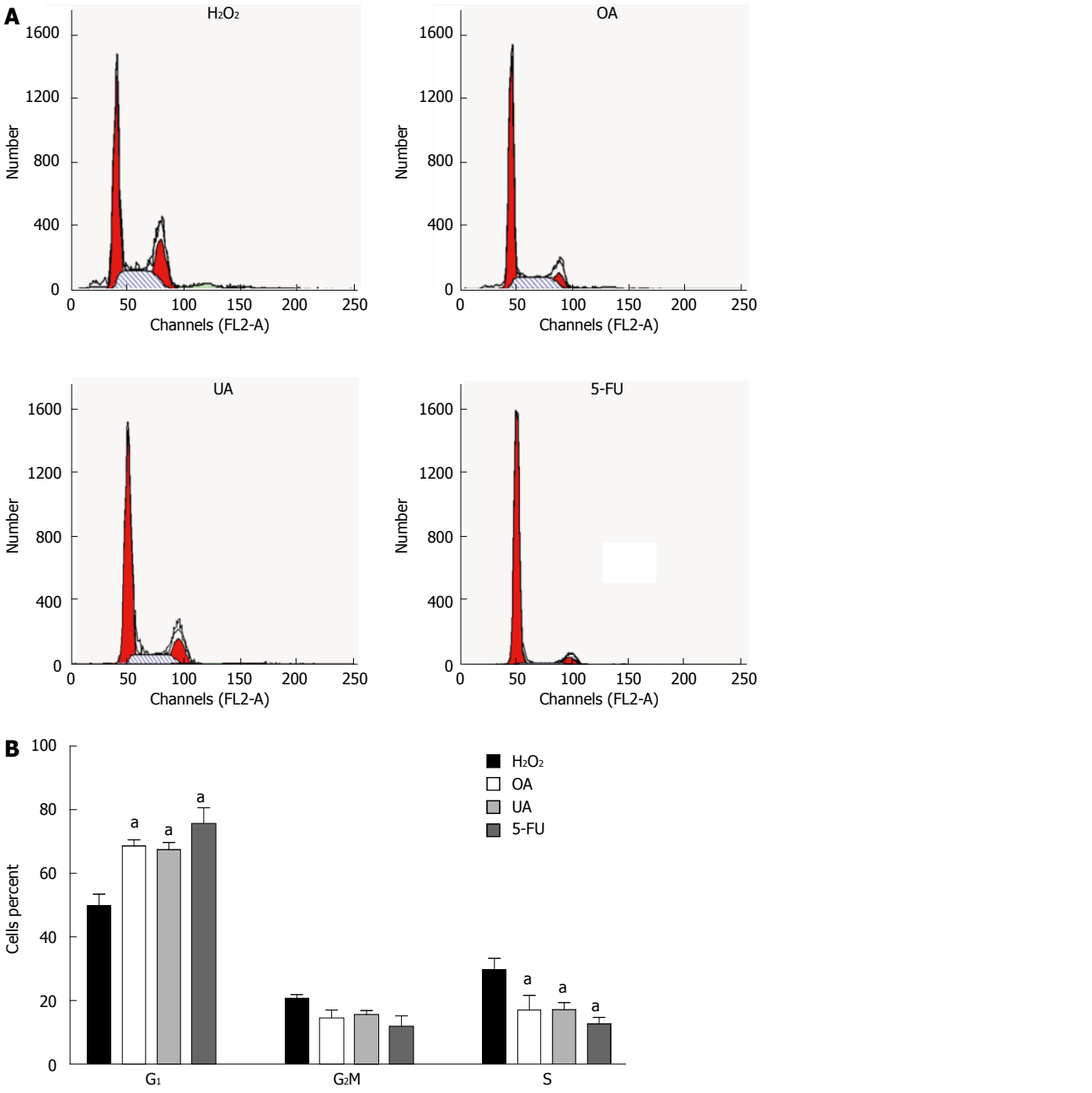

Figure 4 Growth inhibitory effects of oleanolic acid and ursolic acid on H_2O_2-treated WB-F344 cells. A: Flow cytometric analyses of the cell cycle of H_2O_2-treated WB-F344 cells following treatment with oleanolic acid (OA) (4 μmol/L), ursolic acid (UA) (8 μmol/L) or 5-FU (0.05 mmol/L, positive control). After 72 h, the cells were harvested and stained with 10 μg/mL propidium iodide (PI), and the DNA content was analyzed, as described in the Materials and Methods; B: Cell cycle quantitative analysis. Histograms indicating the fraction of H_2O_2-treated WB-F344 cells and those that were treated with OA, UA or 5-FU in the G_1, G_2/M and S phases. The data represent the means ± SD derived from three independent experiments. $^aP < 0.05$ *vs* H_2O_2 model group.

examine the role of OA and UA in hepatic precancerous lesions, we designed experiments to investigate the abnormal proliferation of oval cells in hepatic precancerous lesions.

Cell cycle checkpoint disorders are closely related to the development of tumors. Once the function of the cell cycle monitoring point is weakened, mutant genes can accumulate (*i.e.*, the accumulation of genetic instability). Additionally, the accumulation of mutant genes can destroy the normal progress of the cell cycle, resulting in a vicious cycle. Thus, genetically modified tumor cells are not like normal cells, which are controlled by all cell cycle checkpoints, but instead exhibit uncontrolled growth[29]. One mechanism of anticancer drugs is to control the growth of tumor cells. The present study demonstrated that the cell cycle distribution changed in malignantly transformed WB-F344 cells following UA and OA treatment. The S phase fraction was significantly reduced compared with the control group, and the proportion of cells in G_1 phase was significantly increased, indicating that an inhibition of cell proliferation may be involved in the anti-tumor effects of UA and OA.

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COMMENTS

Background

Hepatocarcinogenesis is a multistage developmental process that is divided into three stages: initiation, promotion and progression. The promotion stage is also referred to as the stage of hepatic precancerous lesions, and its principal pathological feature is oval cell hyperplasia. However, changes in the liver microenvironment can lead to the malignant transformation of oval cells during the process of hyperplasia, which is involved in the initiation and promotion of liver cancer development.

Research frontiers

Oleanolic acid (OA) and ursolic acid (UA) naturally coexist in many plants and herbs and have been reported to possess hepatoprotective effect and anticancer activities. Based on a large number of chemical and pharmacological studies, numerous bioactive compounds have been found in Chinese medicinal plants for prevention and treatment of tumor. OA and UA as monomer drugs showed potential preventive and anti-tumor properties in this study.

Innovations and breakthroughs

It is known that H_2O_2 can promote tumorigenesis. Here, the authors used H_2O_2 as a premalignant and malignant agent to induce proliferation and malignant transformation in quiescent WB-F344 cells. Multistage carcinogenic processes provide the basis for interrupting and reversing precancerous changes; therefore, reversing precancerous changes is critical for tumor prevention and treatment. The salient and novel findings of the study are that OA and UA induced malignantly transformed WB-F344 cell arrest in the G1 phase and apparently inhibited the proliferation of these cells. These results improve their understanding of the anti-tumor effects of OA and UA.

Applications

Hepatocellular carcinoma is one of the most prevalent human malignancies. The prevention and treatment of liver cancer has been receiving a great deal of attention. Traditional Chinese medicines (TCMs), with low toxicity and many targets, have huge advantages. The separation and extraction of effective monomer from TCM is an important direction of anti-tumor drug discovery currently. OA and UA as monomer drugs show potential preventive and anti-tumor properties in hepatocarcinogenesis.

Terminology

Premalignant lesion is a morphologically altered tissue or cells in which cancer is more likely to occur than its apparently normal counterpart. Malignant lesion is a state in which cancer has occurred.

Peer review

The authors induced malignant transformation into rat hepatic oval cells WB-F344 by low-dose H_2O_2 treatment and evaluated the effects of OA and UA. Overall, the data is convincing. The manuscript describes the effect of the phytochemicals OA and UA on the malignant phenotype of tumor cells. The authors analyzed cell morphology and colony formation rates. The data presented in this manuscript is with great interest.

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CASE REPORT

A trichobezoar in a child with undiagnosed celiac disease: A case report

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Abstract

Celiac disease is a chronic, immune-mediated enteropathy caused by a permanent sensitivity to ingested gluten cereals that develops in genetically susceptible individuals. The classic presentation of celiac disease includes symptoms of malabsorption but has long been associated with cognitive, emotional, and behavioral disorders. We describe an 8-year-old patient with non-scarring alopecia and diagnosed with trichotillomania. Furthermore, she presented with a 3-year history of poor appetite and two or three annual episodes of mushy, fatty stools. Laboratory investigations showed a normal hemoglobin concentration and a low ferritin level. Serologic studies showed an elevated tissue immunoglobulin G anti-tissue transglutaminase level. A duodenal biopsy showed subtotal villous atrophy and crypt hyperplasia, and a large gastric trichobezoar was found in the stomach. Immediately after beginning a gluten-free diet, complete relief of trichotillomania and trichophagia was achieved. In this report, we describe a behavioral disorder as a primary phenomenon of celiac disease, irrespective of nutritional status.

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Key words: Celiac disease; Trichobezoar; Trichotillomania; Behavioral disorder; Malabsorption syndrome

Core tip: Celiac disease has been associated with trichotillomania and trichophagia but is always secondary to iron deficiency anemia. We describe a behavioral disorder as a primary phenomenon of celiac disease, irrespective of nutritional status.

Irastorza I, Tutau C, Vitoria JC. A trichobezoar in a child with undiagnosed celiac disease: A case report. *World J Gastroenterol* 2014; 20(5): 1357-1360 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1357.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1357

INTRODUCTION

A trichobezoar is a mass of hair found in the stomach[1] or, less commonly, in the small bowel (Rapunzel syndrome)[2]. This rare condition is nearly exclusively observed in young females. One should be aware of a trichobezoar in young females with psychiatric comorbidity, as this condition is usually the result of the urge to pull out one's own hair (trichotillomania) and swallow it (trichophagia). Human hair is resistant to digestion and peristalsis due to its smooth surface. Hair therefore accumulates among the mucosal folds of the stomach. The continuous ingestion of hair together with mucus and food can cause impaction, leading to the formation of a

trichobezoar[3].

Trichobezoars may not be recognized in the early stages due to their non-specific presentation or even lack of symptoms. When unrecognized, a trichobezoar continues to grow in size and weight due to the continued ingestion of hair. This growth increases the risk of severe complications, such as gastric mucosal erosion, ulceration, and even perforation of the stomach or the small intestine. In addition, intussusception, obstructive jaundice, protein-losing enteropathy, pancreatitis, and even death have been reported in the literature as complications of (unrecognized) trichobezoars[3].

Celiac disease (CD OMIM 212750) is an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically susceptible individuals and characterized by the presence of a variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, an human leukocyte antigen (HLA)-DQ2 or HLA-DQ8 haplotype, and enteropathy. CD-specific antibodies comprise autoantibodies against transglutaminase 2, including endomysial antibodies, and antibodies against deamidated forms of gliadin peptides[4]. This disease is one of the most common lifelong disorders affecting Caucasians. Recent studies have estimated a prevalence of CD close to 1:120[5].

The clinical presentation depends on age, sensitivity to gluten, and the amount of gluten ingested in the diet and on unknown factors. CD has highly variable clinical expression. The classical presentation of CD is characterized by steatorrhea, abdominal distension, and edema, but constitutional symptoms, such as lethargy, poor appetite, depression, and emotional disorders, are frequently present. Iron and folate deficiencies are commonly found, either in isolation or as a feature, and may occur with or without anemia[6].

There are still insufficient data on the association of CD with various neurological disorders in children, adolescents, and young adults, including certain common and "soft" neurological conditions, such as headache, learning disorders, attention-deficit/hyperactivity disorder, and tic disorders[7]. Pica may be a symptom of numerous childhood conditions, including CD[8], and children with this condition require careful diagnostic evaluation.

Only three articles reporting the association of CD and trichobezoars have been found in the medical literature[9-11]. A new case of this rare association is reported.

CASE REPORT

An 8-year-old girl suffering from mild non-scarring alopecia was referred by her pediatrician to a dermatologist. The girl was diagnosed with trichotillomania. Together with this finding, the girl presented with a 3-year history of poor appetite; irritability; clinginess; and two or three annual episodes of mushy, fatty stools. These episodes lasted for one month at most, with normal stools in the meantime. She had a significantly slowed rate of weight and growth during this period. Serologic studies for celiac disease were abnormal, and the girl was therefore referred to the Department of Paediatric Gastroenterology. She weighed 19.5 kg (3rd percentile for age), and her height was 116 cm (below 3rd percentile for age). Her examination revealed pallor, thin hair, areas of alopecia, a decreased subcutaneous adipose layer, muscular hypoplasia, and abdominal distension.

At admission, laboratory investigations showed a normal hemoglobin concentration of 10.9 g/dL [normal range (NR) 10.7-14.8 g/dL], a normal red cell count and hematocrit, mild microcytosis (69.2 fL, NR 73-94 fL), and a normal white cell and platelet count. Iron at 18 mg/dL, ferritin at 10 ng/mL (NR 12-150 ng/mL), transferrin at 348 mg/dL (NR 200-380 mg/dL), and transferrin saturation at 4.1% (NR 20%-40%) were found in ferrokinetic studies. Further investigation revealed an elevated serum glutamic oxaloacetic transaminase level at 93 U/L (NR 7-50 U/L) and a glutamic pyruvic transaminase level at 114 U/L (NR 5-47 U/L), with normal clotting tests.

Serologic studies were repeated and showed a tissue IgA anti-tissue transglutaminase level of 989 U/mL (Celikey®, Phadia, Uppsala, Sweden). To confirm the suspicion of CD, an intestinal biopsy was indicated. A first attempt was performed with a Crosby Capsule, as it was impossible to enter the gastric chamber. An upper endoscopy was performed, and a large gastric trichobezoar was found in the stomach. Attempts to remove the bezoar were unsuccessful, so laparotomy and gastrotomy were therefore performed. By the gastrotomy, a 19 cm by 8 cm gastric bezoar was removed (Figure 1). Duodenal biopsies were obtained to confirm CD. The postoperative course was uneventful.

The patient's duodenal biopsies showed changes consistent with gluten-sensitive enteropathy, including subtotal villous atrophy and crypt hyperplasia (Marsh-Oberhuber 3c). The histological findings, positive tissue IgA anti-tissue transglutaminase antibody testing, and clinical features supported the presumptive diagnosis of CD.

Supplementary iron and a gluten-free diet were instituted. Complete relief of trichotillomania and trichophagia was immediately achieved after the withdrawal of gluten. The excellent response to treatment confirmed the diagnosis of CD. After the patient had been on a gluten-free diet for 6 mo, the antibody levels normalized. Further serologic studies performed to monitor treatment adherence were repeatedly negative. During clinical follow-up, the patient remained asymptomatic, reaching the 50th percentile in weight and height. The control biopsies obtained 2 years after the diagnosis showed a normal intestinal mucosa. After following a gluten-free diet for 4 years, the patient remains asymptomatic, without trichotillomania episodes or other behavioral disorders.

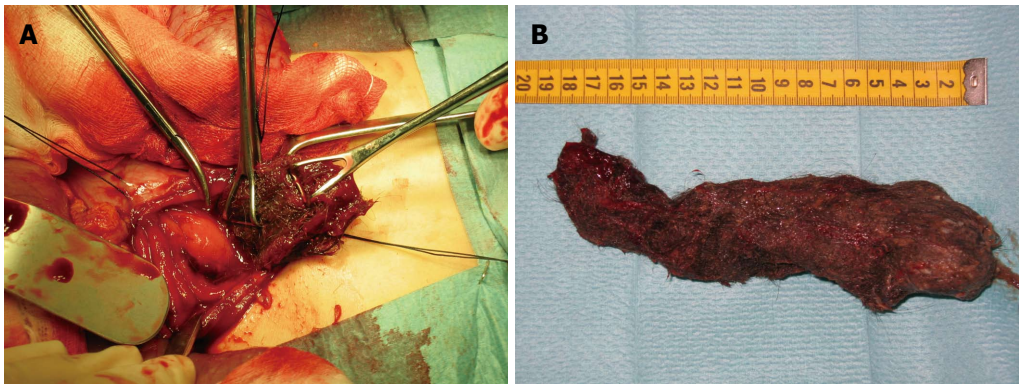

Figure 1 An upper endoscopy was performed, and a large gastric trichobezoar was found in the stomach. A: Attempts to remove the bezoar were unsuccessful, so laparotomy and gastrotomy were therefore performed; B: By the gastrotomy, a 19 cm by 8 cm gastric bezoar was removed. Duodenal biopsies were obtained to confirm celiac disease. The postoperative course was uneventful.

DISCUSSION

Trichotillomania is the morbid craving to pull out hair from the scalp or other parts of the body and is frequently associated with trichophagia, the compulsion to eat hair. This habit is more often observed in girls during the first two decades of life[3]. Trichophagia is one of the various forms of pica (the compulsion to eat non-nutritious substances). The most common underlying conditions associated with pica are mineral deficiency (typically, iron deficiency) and mental health conditions (such as obsessive-compulsive disorder, schizophrenia, and autism). The development of a trichobezoar due to the ingestion of pulled hair is a salient complication.

CD has long been associated with cognitive, emotional, and neurodegenerative disorders, including cerebellar ataxia, peripheral neuropathy, epilepsy, dementia, and depression. One study reported a prior history of psychiatric treatment in a high proportion of adults who were newly diagnosed with CD, even years before the diagnosis[12]. Furthermore, other more frequent neurological manifestations are being researched and may be related to this disease, such as headache, learning disorders, attention-deficit/hyperactivity disorder, and tic disorders[7].

The mechanisms involved in the etiology and pathogenesis of mental and behavioral disorders related to CD are unclear. The cumulative effect of nutritional, inflammatory, or immunologic factors may play a certain role in these manifestations[7]. There are several pathways through acids, and especially tryptophan, may lead to disturbances in central nervous-system serotonin which CD can influence the central nervous system and predispose an individual to mental disorders. Malabsorption of important nutrients, such as folic acid, vitamin B6, and amino function that are associated with depression and aggressive behavior[13].

Case reports on the association of CD and trichophagia have been previously described by three authors[10-12]. In the three patients described, iron deficiency anemia due to intestinal malabsorption appeared to be the primary phenomenon, leading to pica and therefore to trichobezoar formation. There are no reports of pica in patients with CD without anemia.

In this patient, CD presented with severe emotional symptoms. Iron deficiency was detected, but the hemoglobin level was within the normal range. In this patient, trichotillomania was more likely due to behavioral disorders secondary to CD rather than due to the iron deficiency. The fact that this habit disappeared immediately after instituting a gluten-free diet supports this theory.

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CASE REPORT

Nodular fasciitis in the mesentery, a differential diagnosis of peritoneal carcinomatosis

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Abstract

Nodular fasciitis is a benign proliferative lesion composed of fibroblast-like cells that affects various sites in the body. We describe a patient with nodular fasciitis in the mesentery, encountered during laparotomy for the treatment of ascending colon cancer. The nodular fasciitis in our patient resembled peritoneal dissemination of malignancy on macroscopic observation. Because the treatment options change with concomitant peritoneal dissemination of gastrointestinal tract malignancy, recognition of this rare condition and preparation for unexpected nodular lesions are crucial.

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Key words: Colon cancer; Digestive system cancer; Laparoscopic surgery; Soft tissue neoplasms; Neoplasm metastasis; Immunohistochemistry

Core tip: Nodular fasciitis is a rare benign lesion composed of fibroblast-like cells that occurs at various sites in the body. In our patient, firm nodular lesions were incidentally identified in the mesentery during surgical treatment for colon cancer. Although the lesions were initially diagnosed as peritoneal carcinomatosis, the pathological examination revealed nodular fasciitis. This case highlights the need for all clinicians to know about this rare condition and to prepare for unexpected nodular lesions during surgical treatment of gastrointestinal tract malignancy.

Shiga M, Okamoto K, Matsumoto M, Maeda H, Dabanaka K, Namikawa T, Uemura S, Munekage M, Kobayashi M, Hanazaki K. Nodular fasciitis in the mesentery, a differential diagnosis of peritoneal carcinomatosis. *World J Gastroenterol* 2014; 20(5): 1361-1364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1361.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1361

INTRODUCTION

Nodular fasciitis is a rare benign lesion comprising a proliferation of spindle, plump, or stellate fibroblast-like cells embedded in myxomatous stroma[1,2]. Nodular fasciitis involves subcutaneous fat, fascia, and less commonly, areas without true fascia[3,4]. Thus, nodular fasciitis can occur in any part of the body, although the upper extremities or trunk are the sites of predilection[1]. Nodular fasciitis is a reactive fibroblastic growth; however, the majority of patients lack an apparent (recognized) history of injury[1], and its etiology remains unclear.

The classical course of nodular fasciitis varies from stable size to rapid growth[1,2]. Surgical resection is often the treatment for growing nodules and those causing symptoms (such as tenderness and pain), while careful observation of asymptomatic nodules and those that are stable in size is recommended because of possible spontaneous regression[5]. Diagnosis of nodular fasciitis relies largely on the clinical features and careful morphological evaluation of the specimen, including hematoxylin and eosin (HE) staining[6]. Efforts are being made to establish the typical pattern of immunohistochemical staining of nodular fasciitis to help distinguish it from other soft tissue tumors.

Nodular fasciitis is well known to dermatologists and pathologists due to its preferential occurrence in subcutaneous tissue and importance in the differential diagnosis of soft-tissue tumors. Nodular fasciitis is rarely encountered in other fields of clinical practice. In this case report, we describe a patient with nodular fasciitis in the mesentery that mimicked peritoneal dissemination of colon cancer macroscopically.

CASE REPORT

An 88-year-old male patient presented with intermittent gross hematochezia. He had a history of appendectomy for appendicitis about 40 years ago. Preoperative colonoscopy revealed a tumor mass with mild distortion of the intestinal lumen, suggesting tumor infiltration deeper than the submucosal layer. The biopsy confirmed tubular adenocarcinoma of the ascending colon. Computed tomography (CT) and ^{18}F-fluorodeoxy glucose positron emission tomography showed no sign of metastatic lesion or peritoneal dissemination. The preoperative diagnosis was colon cancer, which was staged as IIA (T3, N0, M0) according to the Union for International Cancer Control (UICC) classification[7]. Thus, the patient underwent laparoscope-assisted ileocecal resection with regional lymphadenectomy. After resection of blood vessels and mobilization of the ascending colon, the lesion was removed from the abdominal cavity through a 7-cm incision. A flat and slightly reddish hard nodule about 2 cm in diameter was observed on the mesentery of the terminal ileum (Figure 1A), and a few smaller, whitish nodules were found around it. Peritoneal dissemination of colon cancer was suspected, although intraoperative pathological examination was not considered to change the surgical method because the primary site of the colon cancer had been removed completely. Two large nodules that were suspected of being dissemination of the colon cancer were resected for pathological diagnosis and symptom prophylaxis. Functional end-to-end anastomosis was performed between the terminal ileum and ascending colon.

The postoperative course was uneventful, and the pathological examination revealed sub-mucosal infiltration of colon cancer without lymph node metastasis (Figure 1B) (T1, N0, M0, stage I of the UICC classification[7]). The resected nodule of the mesentery was composed of spindle-like cells in myxomatous stroma without proliferation of adenocarcinoma cells (Figure 1C and D). The nodule was located adjacent to the true muscle layer and there was no encapsulation. The cells of the nodule were positive for vimentin, α-smooth muscle actin (α-SMA), calponin, and negative for podoplanin (D2-40), thrombomodulin, cluster of differentiation molecule 34 (CD34), β-catenin, anaplase lymphoma kinase, S100 protein, cluster of differentiation molecule 56 (CD56), and stem cell factor receptor (c-kit) according to immunohistochemistry staining. The nodule was largely negative for AE1/AE3 and carletinin. A diagnosis of nodular fasciitis was made based on observation of the specimen with HE staining and the immunohistochemical results.

DISCUSSION

This case of nodular fasciitis in the mesentery mimicked peritoneal dissemination of colon cancer, and to the best of our knowledge, is the first such case report. A diagnosis of nodular fasciitis should thus be considered when unexpected nodular lesions suggesting peritoneal dissemination of gastroenterological malignancy are encountered, and such lesions should be treated carefully.

The pathological diagnosis of nodular fasciitis is difficult because of the rarity of the condition, the resemblance of its pathological features to other soft tissue tumors, and the lack of typical diagnostic symptoms or pattern of immunohistochemical staining. In this case, the fibroblast-like cells were arranged haphazardly in the myxomatous stroma, giving a "tissue-culture appearance"[8]. Although the fibroblast-like cells varied in terms of nucleus size and amount of cytoplasm, they differed from the cells found in sarcomas, which typically have higher cellularity, bizarre nuclei, and less intercellular material; therefore, the nodule was considered a reactive proliferative lesion rather than a true tumor. The nodule was positive for vimentin according to immunohistochemical staining, suggesting that the tumor was derived from soft tissue. The majority of nodular fasciitis cases are negative for cytokeratin, with few cytokeratin-positive cases reported[9]. A few spindle cells of nodular fasciitis in our patient were positive for cytokeratin and calretinin, indicating a possible relationship with the mesothelium. Positive staining of α-SMA and calponin suggested myofibroblastic differentiation and less possibility of a sarcoma[6]. The nodule was negative for CD34, CD56, S100 protein, and β-catenin, reducing the possibility of another differential diagnosis (Table 1). A previous report of seven patients demonstrated that approximately half of leiomyosarcoma lesions were negative for cluster of differentiation molecule 10 (CD10) staining[10], while all nodular fasciitis cases were positive for CD10[6]. In our patient, none of the cells expressed CD10, suggesting the need to further evaluate the utility of this marker. Comprehensive judgment based on

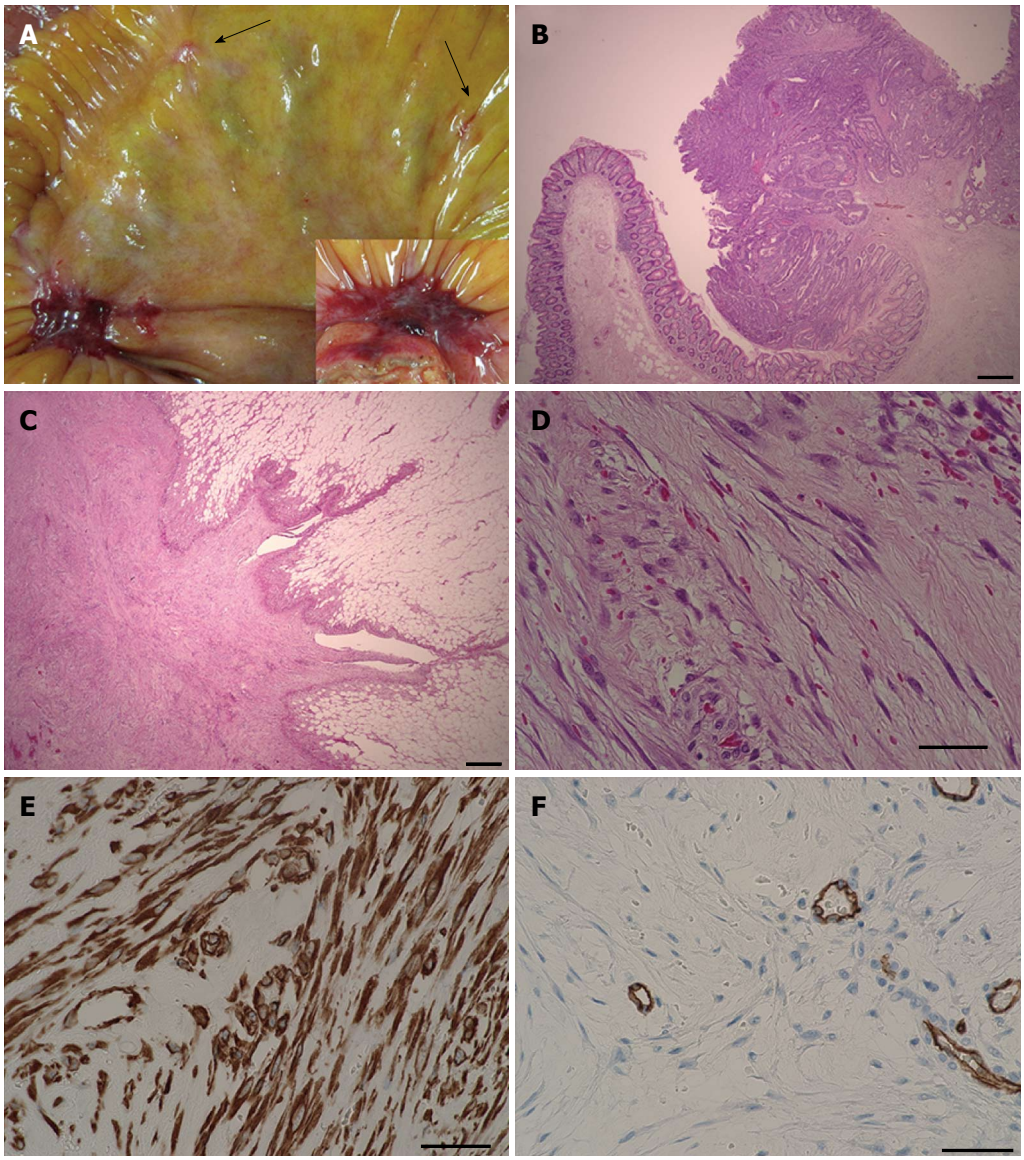

Figure 1 Intraoperative and pathological findings. A: A relatively flat, reddish, and firm nodule was observed on the mesentery of the terminal ilium. The large nodule was accompanied by several whitish, smaller nodules (arrows). The inset shows another resected nodule; B: Resected type 1 ascending colon cancer infiltrating the submucosal layer; C: Lower magnification of nodular fasciitis showing growth in the adipose tissue, radiating outwardly in one part and growing expansively in another part. The margin of the nodule is not clear or encapsulated. Part of the nodule has sparse proliferation of fibroblast-like cells (less-pink area, center), while other parts have a quite dense striform proliferative pattern (pink-red color area); D: Higher magnification of nodular fasciitis demonstrating spindle-shape cells, which are arranged haphazardly throughout the abundant myxomatous interstitial ground substance. Numerous capillaries and extravasation of erythrocytes are visible. Some nuclei are plump, but do not have the hyperchromatic or bizarre pleomorphic features characteristically observed in sarcomas; E: The cytosol of the cells is positive for α-SMA staining, suggesting myofibroblastic differentiation; F: The nodule is negative for CD34, except for endothelial-cell CD34. The mucoid materials on the left side separate the thin spindle-shaped fibroblast-like cells, while the right side of the cells have plump nuclei. The bars for B and C are 500 μm, and the bars for D to F are 50 μm. α-SMA: α-smooth muscle actin.

examination of multiple related markers and close morphological observation is indispensable for the diagnosis of nodular fasciitis.

As many as 10% of patients with colorectal cancer develop peritoneal carcinomatosis during the course of the disease, and half of these are diagnosed at the initial evaluation[11]. While about 20% of T4 tumors are accompanied by peritoneal dissemination, less than 1% of T2 tumors have synchronous peritoneal dissemination. This suggests that less infiltrating tumors have a small, but definite, possibility of peritoneal dissemination, probably occurring *via* a hematological or lymphatic route. While preoperative CT or other imaging modalities may help assess primary tumors, detecting small peritoneal nodules is still difficult, and laparotomy remains an important method for identifying peritoneal dissemination. Ideally, the intraoperative pathological examination for unexpected peritoneal nodules should be performed to inform the choice of surgical methods. When intraoperative pathological diagnosis is not available, judgment regarding surgical options (ranging from observation and biopsy to aggressive surgical resection) must be based on multiple factors including

Table 1 Immunohistochemistry staining for diagnosis of nodular fasciitis

	Nodular fasciitis	Implication
α-SMA	+	Myofibroblastic differentiation
Vimentin	+	Negative in epithelial tumors
Calponin	+	Often negative in myofibroblastic sarcomas
CD10	+	Negative in 50% of leiomyosarcomas
DGP9.5	+	Negative in dermatofibrosarcoma protuberans
AE1/AE3	-	Positive in epithelial tumors
CD56	-	Positive in leiomyomas, nerve sheath tumors, leiomyosarcomas
S100 protein	-	Positive in shwannomas
Desmin	-	Positive in various sarcomas
CD34	-	Positive in solitary fibrous tumors
β-catenin	-	Positive in desmoid fibromatosis

α-SMA: α-smooth muscle actin; CD: Cluster of differentiation molecule.

the patient's condition, tumor spread, and the surgeon's skill. When resection seems inappropriate, biopsy of the nodule for subsequent pathological diagnosis should be performed without exception.

In conclusion, nodular fasciitis in the mesentery can present as unexpected nodules of the peritoneum that resemble peritoneal dissemination of gastrointestinal tract malignancy. It is important to recognize this rare condition because the existence of peritoneal dissemination influences the choice of treatment for gastrointestinal tract malignancy.

COMMENTS

Case characteristics

The patient had no symptoms related to nodular fasciitis.

Clinical diagnosis

Asymptomatic firm nodular lesions were incidentally identified in the mesentery during surgical treatment for colon cancer.

Differential diagnosis

Because the peritoneal nodular fasciitis resembled peritoneal carcinomatosis on macroscopic observation, pathological examination was inevitable.

Imaging diagnosis

The lesions were not identified on the preoperative computed tomography (CT) or ^{18}F-fluorodeoxy glucose positron emission tomography CT.

Pathological diagnosis

Hematoxylin and eosin staining revealed benign proliferation of spindle-like cells in myxomatous stroma, and immunohistochemistry staining suggested the diagnosis of nodular fasciitis.

Treatment

The nodular fasciitis was surgically resected.

Related reports

Nodular fasciitis could occur at any site in the body. However, the case of nodular fasciitis in the abdominal cavity is quite rare.

Experiences and lessons

The nodular fasciitis in our patient closely resembled peritoneal dissemination of colon cancer. Because the treatment options could change with concomitant peritoneal dissemination of gastrointestinal tract malignancy, recognition of this rare condition and preparation for unexpected nodular lesions are crucial.

Peer review

Although nodular fasciitis shows a reactive fibroblastic growth, the majority of patients, including the present patient, lack an apparent history of injury and etiology of nodular fasciitis remains unclear. The further accumulation of clinical experience is necessary to solve this clinical question.

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CASE REPORT

Epstein-Barr virus-associated lymphoepithelioma-like early gastric carcinomas and endoscopic submucosal dissection: Case series

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Abstract

Epstein-Barr virus (EBV)-associated lymphoepithelioma-like gastric carcinoma (LELC) is characterized by a lower lymph node (LN) metastasis rate and a higher survival rate than other forms of gastric cancer. Although current prognosis for LELC is favorable, the most common approach is radical gastrectomy involving an extensive D2 lymph node dissection. Here, we report four cases of EBV-associated early LELC that were treated by an alternative approach, endoscopic submucosal dissection (ESD). The long-term outcome of this procedure is discussed. All patients were treated by ESD *en bloc*, and all ESD specimens showed tumor-free lateral resection margins. None of the lesions showed lymphovascular invasion. A pathological examination of ESD specimens revealed submucosal invasion of more than 500 μm in all four cases. One patient underwent additional radical surgery post-ESD; no residual tumor or LN metastasis was noted in the surgical specimen. The other three patients did not undergo additional surgery, either because of severe comorbidity or their refusal to undergo operation, but were subjected to medical follow-up. None of the ESD-treated patients reported local recurrence or distant metastases during the 27-32 mo of follow-up after ESD.

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Key words: Endoscopic submucosal dissection; Epstein-Barr virus; Lymph node; Lymphoepithelioma-like gastric carcinoma; Prognosis

Core tip: The present case studies indicate that treatment of Epstein-Barr virus (EBV)-associated early lymphoepithelioma-like gastric carcinoma (LELC) by endoscopic submucosal dissection (ESD) might have favorable long-term outcomes, despite deep submucosal invasion of tumor cells. Therefore, a conservative management strategy without additional surgery might be considered for EBV-positive early LELC with submucosal invasion treated by ESD, especially in patients with severe comorbidity and high operative risk.

Lee JY, Kim KM, Min BH, Lee JH, Rhee PL, Kim JJ. Epstein-Barr virus-associated lymphoepithelioma-like early gastric carcinomas and endoscopic submucosal dissection: Case series. *World J Gastroenterol* 2014; 20(5): 1365-1370 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1365.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1365

INTRODUCTION

Lymphoepithelioma-like gastric carcinoma (LELC) is a rare variant of gastric cancer (GC), constituting 1%-4%

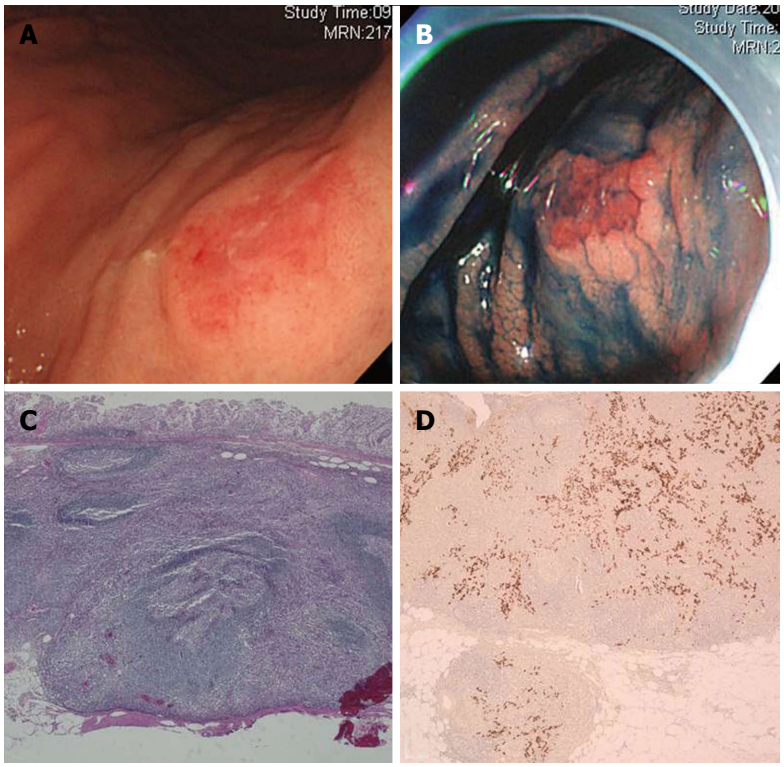

Figure 1 Endoscopic and pathological features of early lymphoepithelioma-like gastric carcinoma treated by endoscopic submucosal dissection (Case 1). A: White light endoscopy image shows early lymphoepithelioma-like gastric carcinoma (LELC) with surface hyperemia; B: Image of the same sample after treatment with indigocarmine dye; C: Photomicrograph of LELC shows dense lymphocytic infiltration and a pushing margin (HE, × 50); D: Photomicrograph of Epstein-Barr virus-encoded RNA detected by in situ hybridization (× 50). Note the positive staining localized specifically to the nuclei of tumor cells, while no signal can be seen in the lymphocytes.

of all GCs[1,2]. More than 80% of the reported LELC cases are associated with an Epstein-Barr virus (EBV) infection. LELC is characterized by (1) a well-defined tumor margin; (2) dense lymphocytic infiltration, with the number of infiltrating lymphocytes exceeding the number of tumor cells; (3) indistinct cytoplasmic borders and a syncytial growth pattern with poorly formed glandular structures; and (4) lack of desmoplasia[3]. When compared to other forms of GC, LELC cases have shown a favorable prognosis, especially those involving an EBV infection[2-5]. This phenomenon could be due to the lower propensity of EBV-associated GC cells invading adjacent lymph nodes (LNs). However, despite the low rate of LN metastasis and a favorable prognosis, current treatment of LELC involves a radical gastrectomy procedure accompanied by extensive D2 LN dissection. Nakamura *et al*[4] reported that the 5-year survival rate of LELC and conventional adenocarcinoma patients undergoing surgical treatment was 84% and 58%, respectively. Few studies have focused on the outcomes of alternative therapeutic strategies for LELC, such as endoscopic submucosal dissection (ESD). Here, we report the long-term outcomes of four cases of EBV-associated early LELC treated with ESD.

CASE REPORT

Case 1

A 63-year-old man reporting poor oral intake underwent esophagogastroduodenoscopy (EGD) to diagnose the cause of his condition. The EGD resulted in the detection of two tumorous lesions in the high body (HB) and low body (LB) of the stomach. The tumor in the HB was a 2.0 cm large round, elevated lesion that displayed surface hyperemia (Figure 1A and B). The lesion in the LB was a 1.0 cm large round, mucosal elevation that displayed a central ulceration. The pathological examination of specimens collected by forceps biopsy of both lesions revealed them to be moderately differentiated adenocarcinomas. A similar biopsy from background mucosa identified the presence of chronic atrophic gastritis and *Helicobacter pylori* (*H. pylori*) infection. No significant enlargement of the LN was detected by a computed tomography (CT) scan of the abdomen. Therefore, ESD was performed on both lesions *en bloc*.

Pathological analysis of the ESD specimen showed that the HB lesion was a 1.6 cm large EBV-positive LELC invading the submucosal layer to a depth of 1800 μm (Figure 1C and D). The LB lesion was characterized as a 0.8 cm large, moderately differentiated adenocarcinoma confined within the mucosa layer. Neither lesion displayed lymphovascular invasion. The resection margins of both lesions were tumor-free. Since the LELC lesion showed deep submucosal invasion (1800 μm), the patient underwent total gastrectomy following ESD. No residual tumor or LN metastasis was observed in the surgical specimen after completion of the surgical treatment. In addition, no signs of recurrence were detected during the first 48 mo of follow-up post gastrectomy.

Case 2

A 65-year-old man was referred to our hospital due to an incidentally found early GC on screening EGD. A second EGD performed in our hospital revealed slightly elevated 2.0 and 1.0 cm large lesions in the LB (Figure 2A) and mid body (MB) of the stomach, respectively. Analysis of specimens collected by forceps biopsy of the LB and MB lesions enabled classification of the two lesions as a moderately differentiated adenocarcinoma and a high-

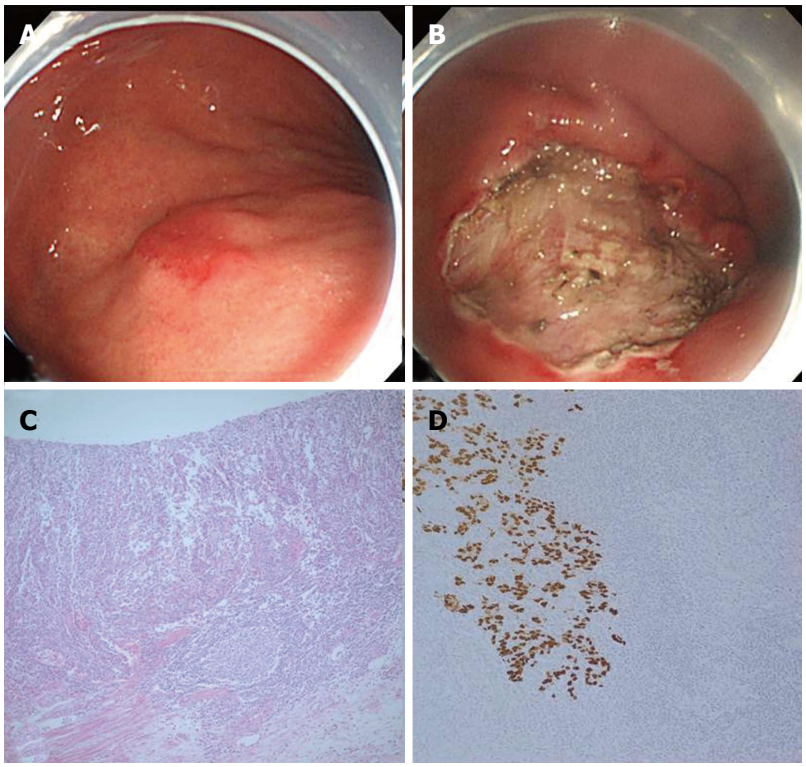

Figure 2 Endoscopic and pathological features of early lymphoepithelioma-like gastric carcinoma treated by endoscopic submucosal dissection (Case 2). A: White light endoscopy image shows a slightly elevated lesion with surface hyperemia; B: Appearance of the iatrogenic ulcer after ESD; C: Photomicrograph of lymphoepithelioma-like gastric carcinoma showing a dense infiltration of lymphocytes in the tumor stroma (HE, × 50); D: In situ hybridization of Epstein-Barr virus-encoded RNA in carcinoma cells (× 50).

grade dysplasia, respectively. A similar pathological review of the background mucosa identified the presence of chronic atrophic gastritis and *H. pylori* infection. CT scan of the abdomen detected no definite enlargement of the LNs. ESD was thus performed on both lesions *en bloc*, and ESD specimens were further examined. Consequently, the LB lesion was described as a mixture of a 1.8 cm large well-differentiated adenocarcinoma within the mucosa layer, and a 0.6 cm large EBV-associated LELC with submucosal invasion of up to 1800 μm (Figure 2C and D). The MB lesion was characterized as a 0.8 cm large high-grade dysplasia. Neither lesion displayed lymphovascular invasion. The resection margins of both lesions were tumor-free. To treat the deep submucosal invasion of the LELC tumor, an additional radical surgery accompanied by LN dissection was recommended. However, the patient refused to undergo another operation, but requested medical follow-up. During the 32 mo of follow-up after ESD, no signs of local recurrence or distant metastases were found.

Case 3

A 74-year-old man underwent EGD as part of a routine check-up. A 1.5 cm large, slightly elevated lesion with central dimpling was detected in the LB of the stomach. The lesion was covered with normal-looking mucosa and mimicked a subepithelial tumor (Figure 3A). Endoscopic ultrasound (EUS) revealed a homogeneous hypoechoic lesion growing from the muscularis mucosa layer, which also seemed to invade into the thickened submucosa (Figure 3B). Pathological examination of specimens collected by forceps biopsy identified a few markedly atypical cells in the submucosa, suggesting that the lesion could be an undifferentiated carcinoma. Chronic atrophic gastritis was found in the background mucosa. Unlike the previous cases, *H. pylori* infection was not detected. Results from the CT scan of the abdomen were inconclusive. To obtain a definite pathological diagnosis, ESD was performed *en bloc* (Figure 3C). Analysis of the ESD specimen showed a 1.8 cm large EBV-positive LELC invading the submucosal layer to a depth of 2500 μm (Figure 3D-F). No lymphovascular invasion was detected. Resection margins of the lesion were found to be tumor-free. Due to the lesion's deep submucosal invasion, an additional radical surgery along with LN dissection was recommended. However, the patient refused to undergo another operation, but did agree to medical follow-up. During this 28-mo period following ESD, neither EGD nor abdominal CT scan detected signs of local recurrence or distant metastases.

Case 4

An 84-year-old man underwent EGD as part of a regular check-up. He had received concurrent chemoradiotherapy for treating a marginal zone B cell lymphoma of the hard palate two years ago, and was in complete remission at the time of the EGD. The EGD revealed a 1.5 cm large reddish and slightly depressed lesion in the cardia (Figure 4A and B), later characterized to be a moderately differentiated adenocarcinoma based upon examination of specimens collected by forceps biopsy. Chronic atrophic gastritis, but no *H. pylori* infection, was detected in the background mucosa. Abdominal CT scan identified no definite enlargement of the LNs. The patient was treated by ESD *en bloc*. Pathological analysis of the ESD specimen indicated the lesion was a 2.2 cm large EBV-

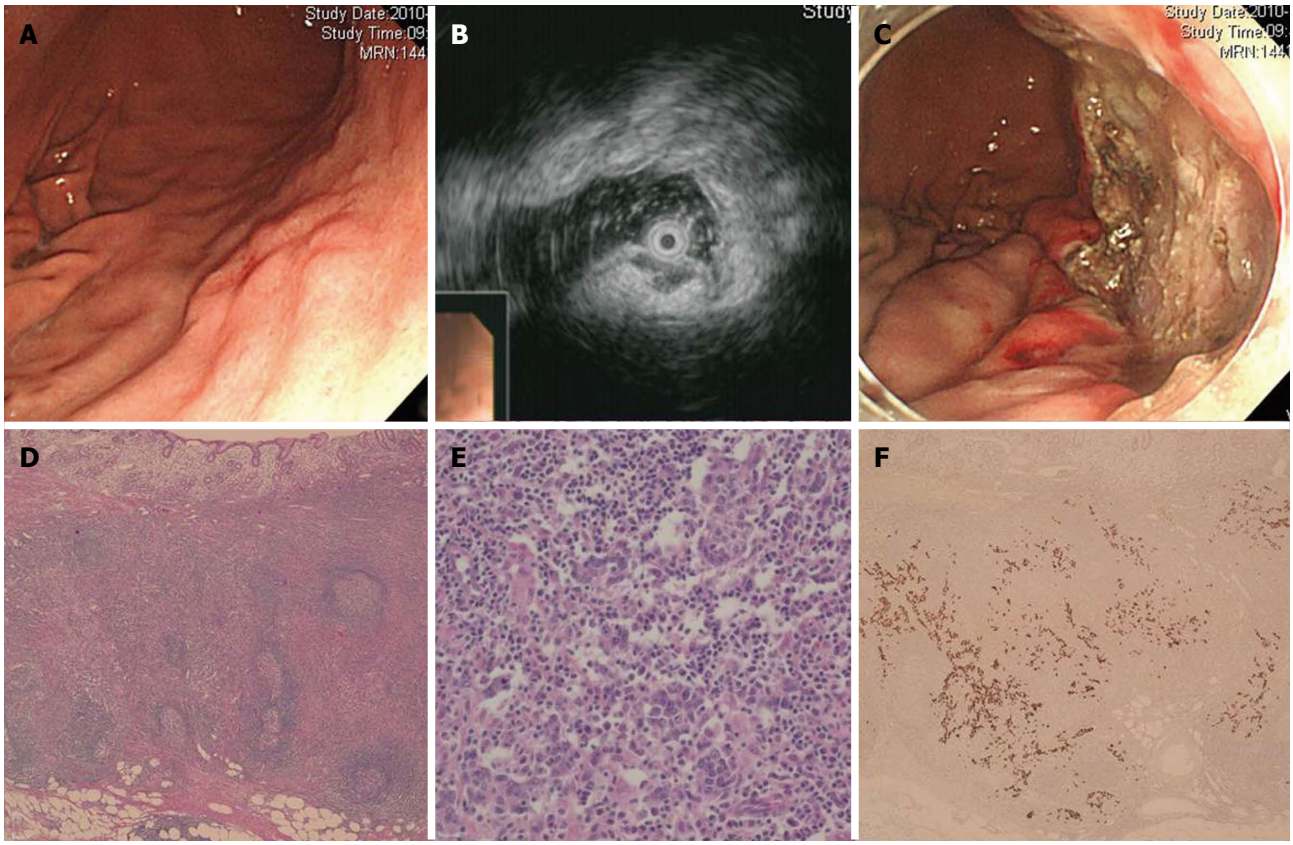

Figure 3 Endoscopic and pathological features of early lymphoepithelioma-like gastric carcinoma (mimicking a subepithelial tumor) treated by endoscopic submucosal dissection (Case 3). A: White light endoscopy image shows early lymphoepithelioma-like gastric carcinoma (LELC) mimicking a subepithelial tumor; B: Endoscopic ultrasound image shows a homogeneous hypoechoic lesion growing from the muscularis mucosa. Invasion of this lesion into the thickened submucosa layer can also be seen; C: Appearance of the iatrogenic ulcer after endoscopic submucosal dissection; D: Photomicrograph of LELC shows a dense infiltration of lymphocytes in the tumor stroma (HE, × 50); E: Nests of tumor cells separated by a dense infiltration of lymphocytes (HE, × 400); F: *In situ* hybridization of Epstein-Barr virus-encoded RNA in carcinoma cells (× 50).

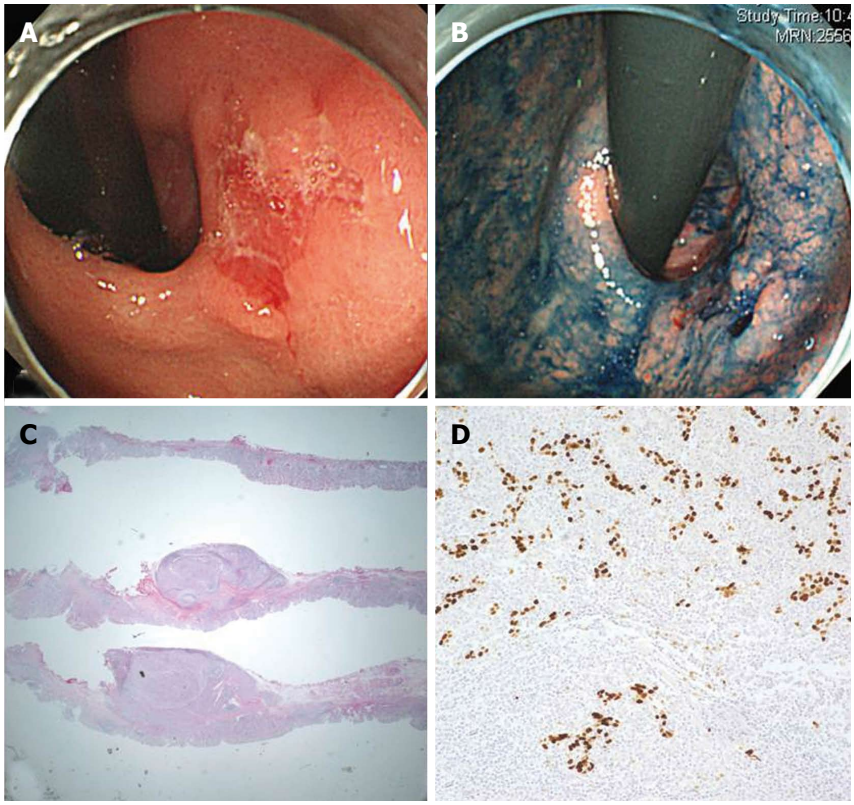

Figure 4 Endoscopic and pathological features of early lymphoepithelioma-like gastric carcinoma treated by endoscopic submucosal dissection (Case 4). A: White light endoscopy image shows a reddish and slightly depressed lesion in the cardia; B: Image of the same sample after treatment with indigocarmine dye; C: Photomicrograph of lymphoepithelioma-like gastric carcinoma shows dense lymphocytic infiltration and a pushing margin (HE, × 4); D: Photomicrograph of Epstein-Barr virus (EBV)-encoded RNA detected by *in situ* hybridization (× 50).

associated LELC, with submucosal invasion of up to 2300 μm (Figure 4C and D). While lymphovascular invasion was not observed, the deep resection margin of the lesion was found to be invaded by tumor cells; the presence of a marked cautery artifact was also noted. Hence, an additional gastrectomy procedure was considered. However, the patient's old age and poor pulmonary function (with forced expiratory volume of 1.09 L) complicated the radical surgery option, and after consulting with the patient the approach was decided against. Instead, the patient was closely monitored by EGDs and abdominal CT scans. During the 27 mo of follow-up after ESD, no signs of local recurrence or distant metastases were detected.

DISCUSSION

ESD is an effective therapeutic strategy for early GC, and is recognized as the preferred method of treatment in selected cases. Studies have demonstrated that the *en bloc* resection rate of ESD is greater than 90%, regardless of tumor size, and that the long-term survival rate following ESD is comparable to that of radical gastrectomy. The reported rates of postoperative bleeding and perforation range from 3.4%-7.6% and 1.0%-6.1%, respectively[6]. Few studies to date have examined the long-term outcomes of ESD treatment of early LELC[7,8]; among these, detailed pathological features of LELC have been described in only one case report. Lee *et al*[8] reported a case of EBV-associated LELC with submucosal invasion of up to 1538 μm, which was completely resected by ESD. No additional radical surgery was performed. During 24 mo of follow-up using EGD no local recurrence was detected. Our own cases of EBV-positive LELCs also showed favorable long-term outcomes following ESD, despite deep submucosal invasion of more than 500 μm. This favorable prognosis may be related to a lower propensity of EBV-associated GC to invade adjacent LNs, perhaps due to the immune response mounted against EBV proteins expressed by tumor cells[5]. LELC is characterized by a dense infiltration of lymphocytes in the tumor stroma. This high degree of infiltration could be another reason for the favorable prognosis. Song *et al*[3] reported that patients with EBV-associated LELC or GC accompanied by a Crohn's disease-like lymphocyte reaction show significantly longer overall and disease-free survival following radical surgery than those with EBV-positive conventional adenocarcinoma. This in turn strongly suggests the importance of the host's inflammatory response in determining the prognosis of EBV-associated GC. Nakamura *et al*[4] also reported that the 5-year overall survival rate following surgical treatment is significantly higher in patients diagnosed with LELC (84%) compared to those with conventional adenocarcinoma (58%).

The endoscopic features of LELC have not been well described. Several studies have reported cases of LELC histology mimicking features of subepithelial tumors[7,9,10]. Although rare, these studies have demonstrated the existence of hypoechoic lesion in the submucosa and the muscularis mucosa. This hypoechoic lesion might correspond to the lymphoid stroma composed of carcinoma cells and infiltrating lymphocytes. Results from EUS of Case 3 in our study corroborated the above findings (Figure 3B). Because of the dense lymphocytic aggregations and scattered foci of subepithelial neoplastic cells in LELC cases mimicking subepithelial tumors, pathological diagnosis of cancer using direct tissue sampling methods, such as fine needle aspiration is challenging. Therefore, ESD should be considered as a tool for a definite diagnosis and appropriate management of lesions if a lesion with endoscopic features of subepithelial tumor shows atypical cells in forceps biopsy specimen and above-mentioned findings in EUS.

In summary, our case series shows that ESD treatment of EBV-associated early LELC might have favorable long-term outcomes, despite deep submucosal invasion of tumor cells. Therefore, a conservative management strategy without additional surgery might be considered for EBV-positive early LELC with submucosal invasion treated by ESD, especially in patients with severe comorbidity or high operative risk. To enable conservative management, intensive medical follow-up should be performed using EGDs and abdominal CT scans. One shortcoming of this report is the limited number of cases; to confirm our findings, data from a larger number of case studies needs to be collected and analyzed.

COMMENTS

Case characteristics

Only one patient underwent esophagogastroduodenoscopy (EGD) to diagnose the cause of poor oral intake; the other three patients underwent EGD as part of a routine check-up, and did not display any symptoms requiring examination.

Clinical diagnosis

Since three of the patients were asymptomatic and only one patient complained of a non-specific symptom, clinical diagnosis of prior to endoscopic submucosal dissection (ESD) was difficult.

Differential diagnosis

From EGD findings, early gastric cancer (GC) was diagnosed in three cases; the fourth patient was suspected to have a subepithelial tumor.

Laboratory diagnosis

From the examination of specimens collected by forceps biopsy, three cases were diagnosed as moderately differentiated adenocarcinomas and one was described as a condition with few markedly atypical cells in the submucosa.

Imaging diagnosis

No definite enlargement of the lymph nodes was detected in any of the four cases, according to CT scanning of the abdomen.

Pathological diagnosis

Pathological examination of ESD specimens revealed dense lymphocytic infiltration in the tumor stroma. *In situ* hybridization detected Epstein-Barr virus (EBV)-encoded RNA in carcinoma cells.

Treatment

ESD was successfully performed *en bloc* for all four cases.

Term explanation

LELC is a rare variant of GC characterized by a dense infiltration of lymphocytes in the tumor stroma.

Experiences and lessons

The case series shows that ESD treatment of EBV-associated early LELC might have favorable long-term outcomes, despite deep submucosal invasion of tumor cells.

Peer review

This report will help other clinicians in determining the prognosis of early LELC with deep submucosal invasion following an ESD treatment. To confirm the findings reported here, information from more case studies needs to be collected and analyzed.

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CASE REPORT

Bowel mesentery (meso-appendix) microcystic/reticular schwannoma: Case report and literature review

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Abstract

Microcystic/reticular schwannoma is a recently described variant of schwannoma with a predilection for the gastrointestinal tract. Due to overlapping features with other tumors, unawareness of this tumor type may lead to diagnostic and therapeutic pitfalls. We here report a case of microcystic/reticular schwannoma arising in the meso-appendix of a 43-year-old woman. The tumor was incidentally discovered by computed tomography scan for unrelated reasons. A laparoscopic operation was performed shortly after admission. Histological examination revealed a circumscribed tumor with a striking microcystic and cribriform architecture. Immunohistochemically, the tumor cells were diffusely positive for S100 protein, glial fibrillary acid protein and protein gene product 9.5, which were consistent with a peripheral nerve sheath tumor. The patient remains well with no signs of recurrence at a 10-mo follow-up. To our knowledge, this is the first case of microcystic/reticular schwannoma arising in the meso-appendix. Albeit very rare, microcystic/reticular schwannoma should be included in the differential diagnosis of appendiceal tumors.

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Key words: Laparoscopy; Schwannoma; Appendiceal neoplasms; Immunohistochemistry; Differential diagnosis; Gastrointestinal tract

Core tip: Microcystic/reticular schwannoma is a recently described variant of schwannoma with a predilection for the gastrointestinal (GI) tract. The striking reticular growth pattern and myxoid background may cause confusion with several other tumors commonly seen in the GI tract, in particular a gastrointestinal stromal tumor with prominent myxoid change and a signet ring cell carcinoma, especially on small biopsies. Herein we report for the first time a microcystic/reticular schwannoma arising primarily in the meso-appendix to highlight its existence and enhance pathologist's and clinician's awareness of this under-recognized variant of schwannoma so as to avoid misdiagnosis and mistreatment.

Tang SX, Sun YH, Zhou XR, Wang J. Bowel mesentery (meso-appendix) microcystic/reticular schwannoma: Case report and literature review. *World J Gastroenterol* 2014; 20(5): 1371-1376 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i5/1371.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i5.1371

INTRODUCTION

Schwannoma usually arises in the subcutaneous tissue of the distal extremities or the head and neck region of adult patients with no sex predilection. Occasionally, the tumor may also involve a wide variety of anatomic

Figure 1 Computed tomography scan of the abdomen revealing a well-circumscribed mass (arrow) with isodensity located distal to the appendix.

sites, including the mediastinum, gastrointestinal tract, retroperitoneum, spinal cord, cerebellopontine angle and bone. Apart from those arising in the setting of neurofibromatosis type 2 (NF2), most schwannomas are solitary and sporadic lesions, although a small percentage of cases are associated with von Recklinghausen's disease. The typical schwannoma is characterized by a biphasic pattern composed of alternating Antoni A and Antoni B areas. In addition to the classic type, approximately 11 morphological variants have been recognized. These variants include ancient (degenerated) schwannoma[1], cellular schwannoma[2], plexiform schwannoma[3], melanotic schwannoma[4,5], epithelioid schwannoma[6], hybrid schwannoma/neurofibroma[7], hybrid schwannoma/perineurioma[8], gastrointestinal schwannoma[9], neuroblastoma-like schwannoma[10], lipoblastic schwannoma[11], and microcystic/reticular schwannoma[12]. Frankly speaking, these variants simply represent the morphological heterogeneity in schwannoma with no distinct relationship to the clinical behavior. Nevertheless, unawareness of these rare variants will lead to diagnostic pitfalls and risk of mistreatment of the patients. We present here a case of microcystic/reticular schwannoma arising in the vermiform appendix, a unique site that has not been described before. The clinical and pathological features of all 13 cases of microcystic/reticular schwannoma originated in the gastrointestinal tract were summarized.

CASE REPORT

Clinical findings

A 43-year-old woman presented with a pelvic mass which was incidentally discovered by computed tomography (CT) scan during a check-up for mild abdominal pain. On physical examination, the abdomen was soft and flat with no tenderness on deep palpation and no palpable mass could be identified. However, CT scan revealed an isodense mass in the right lower quadrant, measuring 4.5 cm × 3.4 cm × 4.6 cm in size (Figure 1). Slow and persistent enhanced signal was observed following administration of contrast. Her medical history was unremarkable. There was no clinical manifestation of either neurofibromatosis type 1 (NF1) or type 2 (NF2). With a suspicion of a gastrointestinal stromal tumor (GIST) or a leiomyoma, the patient was admitted for scheduled surgery. During laparoscopic operation, a solid well-circumscribed mass was found in the distal end of the meso-appendix, measuring approximately 4 cm in diameter. The patient recovered uneventfully after surgery and is now well with no evidence of recurrence at a 10-mo follow-up.

Pathological findings

The submitted specimen consisted of white to gray fragmentary tissues measuring 4.0 cm × 4.0 cm × 1.9 cm in volume. It had a homogeneous gelatinous texture, showing no features of necrosis, hemorrhage or cystic degeneration. At scanning magnification, the tumor was well circumscribed and surrounded by a thin fibrous capsule (Figure 2A). It was composed of relatively alternating fibrillary and myxoid areas (Figure 2B). There was transition between these two areas. Tumor cells were spindle-shaped with eosinophilic cytoplasm and ovoid or tapered nuclei harboring small inconspicuous nucleoli. They had generally bland appearance without nuclear pleomorphism or degenerative atypia. Mitotic figure was scarce with less than 3/50 HPF. In fibrillary areas, the tumor cells were generally arranged in irregular fascicles with no palisading or Verocay body formation, whereas in myxoid areas, they were arranged in anastomosing or intersecting strands creating a striking lace-like or microcystic growth pattern (Figures 2C and D). In focal areas, remarkable perivascular lymphocytic aggregates were present.

Immunohistochemistry was performed on paraffin-embedded sections using the standard EnVision method. The tumor cells showed diffuse and strong nuclear and cytoplasmic staining of S100 protein (Figure 3A). They were also positive for glial fibrillary acid protein (GFAP) and protein gene product 9.5 (PGP9.5) (Figure 3B). Focal staining was also found with CD117. MIB-1 showed low proliferative activity with index less than 1%. A few CD34 positive cells were observed among the stromal cells, indicating the presence of fibroblast or perineurial cells within the tumor. Tumor cells were all negative for CD57, DOG1, desmin, alpha smooth muscle actin, pancytokeratin (AE1/AE3), epithelial membrane antigen (EMA), synaptophysin, CD31 and P53.

We did sequencing analysis of exons 9, 11, 13, and 17 of *c-KIT* gene to exclude the possibility of GIST. Polymerase chain reaction assays were carried out using oligonucleotide primer pairs as previously described. Direct sequencing was performed on the ABI Prism 310 DNA sequencer. The result demonstrated that the tumor did not harbor c-*KIT* gene mutation.

DISCUSSION

Microcystic/reticular schwannoma is a rare variant of schwannoma. Including the current case, only 22 cases have been reported in the literature[12-21]. Due to its rarity, this tumor type has remained under-recognized. As

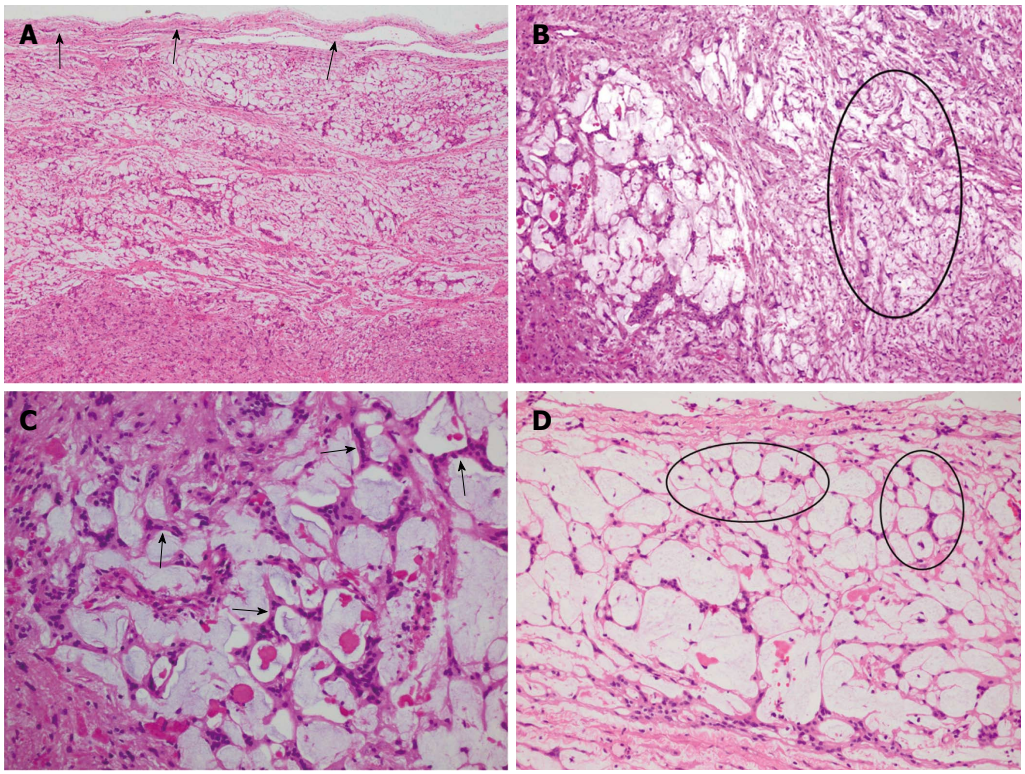

Figure 2 Pathological findings (Hematoxylin-eosin staining). A: Low power showing a thin fibrous capsule at the periphery (arrow) (× 20); B: Prominently myxoid areas (ellipse) (× 40); C: High magnification illustrating lacelike (arrow) (× 200); and D: Microcystic (ellipse) (× 100) arrangement of tumor cells.

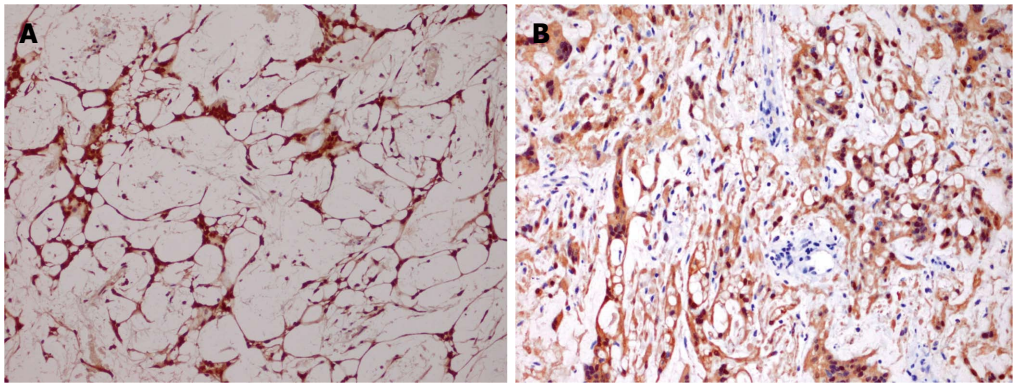

Figure 3 Immunohistochemical staining (EnVision method). A: S100 protein (× 100); B: Protein gene product 9.5 (× 100).

there are overlapping features with a wide variety of neoplasms, this tumor type may be misdiagnosed and potentially lead to mistreatment of the patients. Therefore, enhanced awareness of its characteristic features is imperative for pathologists to avoid diagnostic pitfalls. To enhance the recognition, we present here an additional case of microcystic/reticular schwannoma and undertake a brief review of the literature.

Taking the current case into account, there are 13 cases of microcystic/reticular schwannoma arising primarily in the gastrointestinal tract, accounting more than a half of all cases. The overall median and average ages of patients with gastrointestinal microcystic/reticular schwannoma at diagnosis were 68 years and 66 years, respectively (range, 32-93 years). There was a predilection for female patients, with a male/female ratio of 1:3.3 (Table 1). Clinically, the majority of patients presented with an asymptomatic mass discovered incidentally by imaging examinations during routine check-up or at operation for other unrelated reasons. A few patients complained of indigestion, bowel habit change or epigastric pain[13,16]. Occasionally, the tumor appeared as an intestinal polypoid lesion at colonoscopy[12,14]. To date, none has clinical evidence of NF1 or NF2. The tumors ranged in size from 0.4 to 4.0 cm (median, 1.3 cm; mean, 1.5 cm). With regard to the site, 4 cases occurred in the colon, 3 cases in the small intestine, 2 cases in the cecum, 2 cases in the stomach, and 1 case each in the rectum and meso-appendix.

Except for the 4 tumors showing focal infiltration

Table 1 Summary of patients with gastrointestinal microcystic/reticular schwannoma

Ref.	Sex/age (yr)	Location	Size (cm)	Symptoms	Gross appearance	Outcome (mo)
Liegl *et al*[12]	F/73	Rectum	0.85	Asymptomatic	Circumscribed, not encapsulated	Died at 36[1]
Liegl *et al*[12]	F/72	Stomach	2.0	Asymptomatic	Circumscribed, not encapsulated	ANED at 24
Liegl *et al*[12]	M/68	Cecum	0.4	Asymptomatic	Circumscribed with focal infiltration	ANED at 24
Liegl *et al*[12]	F/93	Jejunum	1.6	Asymptomatic	Circumscribed, not encapsulated	ANED at 7
Liegl *et al*[12]	M/78	Small intestine	0.8	Asymptomatic	Circumscribed with focal infiltration	"Recent case"
Lee *et al*[13]	F/32	Ascending colon	1.4	Bowel habit change	Circumscribed with focal infiltration	UA
Agaimy *et al*[14]	F/67	Cecum	1.0	A polyp at colonoscopy	Circumscribed with focal infiltration	ANED at 12
Agaimy *et al*[14]	F/67	Mid-jejunum	2.2	Incidental finding	UA	ANED at 2
Kienemund *et al*[15]	F/70	Sigmoid colon	0.7	UA	Circumscribed, not encapsulated	UA
Kienemund *et al*[15]	F/70	Sigmoid colon	1.3	UA	UA	UA
Chetty *et al*[16]	F/63	Stomach	1.9	Epigastric pain	Circumscribed, not encapsulated	ANED at 60
Trivedi *et al*[18]	M/61	Sigmoid colon	0.7	Incidental finding	Circumscribed, not encapsulated	ANED at 24
Our case	F/43	Meso-appendix	4.0	Incidental finding	Circumscribed and encapsulated	ANED at 10

[1]Died of metastatic colon carcinoma; ANED: Alive with no evidence of disease; UA: Unavailable.

between smooth muscle fibers or extending into the mucosa[12-14], the other gastrointestinal microcystic/reticular schwannomas were all well circumscribed. On histology, microcystic/reticular schwannoma differs from a classic schwannoma in many ways. The former lacks the distinctive features that are typically noted in a classic schwannoma, namely alternating areas of Antoni A and Antoni B, presence of palisading or Verocay bodies, aggregates of foamy histiocytes, and hyalinized blood vessels. The hallmark of microcystic/reticular schwannoma is the presence of a striking reticular and microcystic architecture, a feature not observed in any other variants of schwannoma.

Regardless of the preferential location in the gastrointestinal tract, microcystic/reticular schwannoma is different from another variant of so-called gastrointestinal schwannoma[9]. Gastrointestinal schwannoma is relatively more common than microcystic/reticular schwannoma. This type of schwannoma tends to occur in the stomach, although intestines can be occasionally involved. Histologically, it is composed of spindled Schwann cells displaying frequently a microtrabecular or microfascicular pattern and characterized by a peritumoral lymphocytic cuff. Although focal myxoid change can be observed in a few gastrointestinal schwannomas, prominent microcystic pattern is never seen.

Besides variants of schwannoma, there are other types of benign peripheral nerve sheath tumor occurring in the gastrointestinal tract which may cause confusion among them. These tumors include perineurioma and hybrid schwannoma/perineurioma. Perineurioma is composed of bland spindle cells with long bipolar cytoplasmic processes embedded in fine collagenous stroma which sometimes appears myxoid[22]. The typical feature of "pseudo-onion bulb" arrangement of tumor cells around a central axon will help arrive at a correct diagnosis. However, the reticular variant of perineurioma may present diagnostic dilemma. This unusual morphologic variant occurs preferentially in the soft tissue with no case of gastrointestinal origin documented[23]. Immunostaining with a panel of antibodies including EMA, claudin-1, S100 protein and GFAP will facilitate the differential diagnosis. Hybrid schwannoma/perineurioma is a benign tumor consisting of intimately mixed components of plump spindled Schwann cells and slender perineurial cells. This novel type of peripheral nerve sheath tumor can also occur in the gastrointestinal tract[8]. However, tumor cells are frequently arranged in a storiform or fascicular pattern and the stroma rarely undergoes myxoid change. Double immunostaining of S100 protein and EMA can clearly highlight the two components.

Occasionally, GIST may have myxoid change. Although the majority of GIST arise in stomach and intestines, involvement of appendix is not uncommon[24]. Indeed, it has been acclaimed that GIST represents the most common type of appendiceal mesenchymal tumors[25]. As the treatment varies greatly, a distinction between GIST and microcystic/reticular schwannoma is warranted. Appendiceal GISTs are usually incidental findings. Some cases may masquerade as appendicitis. Microscopically, they are indolent tumors composed of spindle cells with prominent extracellular collagen globules, known as skeinoid fibers. Immunohistochemically, appendiceal GISTs are ubiquitously positive for CD117

and DOG1, whereas the staining of S100 protein is consistently negative. It is worthy to note that focal immunoreactivity of CD117 can be observed in a minority of microcystic/reticular schwannoma. However, the absence of *KIT* or *PDGFRA* gene mutation denies the diagnosis of GIST.

As abovementioned, most cases of microcystic/reticular schwannoma were well circumscribed. However, a few tumors showed focal infiltration between the smooth muscle fibers of the muscularis mucosa, between the colonic crypts, or extended into the mucosa[12-14]. The epithelioid morphology, signet ring cell appearance of the tumor cells in some cases together with a myxoid background may cause confusion with poorly differentiated adenocarcinoma or signet ring cell carcinoma[12,18]. Of note, the so-called signet ring cell gastric schwannoma described by Tozbikian *et al*[26] in 2008, in our opinion, represents a morphologic spectrum of microcystic/reticular schwannoma. Erroneous diagnosis of a benign microcystic/reticular schwannoma as a malignant signet ring cell carcinoma will lead to inappropriate treatment of the patients. The absence of nuclear atypia and negativity for epithelial markers allow the differentiation of microcystic/reticular schwannomas from carcinomas.

In summary, we reported for the first time a microcystic/reticular schwannoma arising primarily in the vermiform appendix. Although very rare, microcystic/reticular schwannoma represents a unique and distinctive morphological variant of schwannoma with a benign clinical course. Enhanced awareness of its characteristic features will facilitate the differential diagnosis from a wide variety of neoplasm with overlapping features.

COMMENTS

Clinical diagnosis

The patient was incidentally found to have a pelvic mass by computed tomography (CT) scan during a check- up for mild abdominal pain.

Differential diagnosis

The characteristic features in immunohistochemistry may facilitate the differentiation of microcystic/reticular schwannoma from a variety of tumors with overlapping features.

Laboratory diagnosis

Immunohistochemically, the tumor cells showed diffuse and strong nuclear and cytoplasmic staining of S100 protein and glial fibrillary acid protein (GFAP), compatible with a peripheral nerve sheath tumor.

Imaging diagnosis

CT scan revealed an isodense mass in the right lower quadrant which was suspected to be a gastrointestinal stromal tumor (GIST) or a leiomyoma.

Pathological diagnosis

The well-circumscribed tumor was composed of relatively alternating fibrillary and myxoid areas, creating a distinctive lacelike and microcystic growth pattern.

Treatment

At laparoscopic surgery, the solid mass of the meso-appendix was excised and no adjuvant treatment was applied after surgery.

Related reports

Thirteen cases of gastrointestinal microcystic/reticular schwannoma have been reported, most of which are located in the wall of the gastroenterologic tract. This is a first case of a microcystic/reticular schwannoma arising primarily in the vermiform appendix.

Term explanation

Although termed as a schwannoma, the tumor lacks the distinctive features that are typically noted in a classic schwannoma, such as alternating areas of Antoni A and Antoni B, presence of palisading or Verocay bodies, aggregates of foamy histiocytes, and hyalinized blood vessels. The hallmark of this rare variant is the presence of a striking reticular and microcystic architecture which may cause confusion with other neoplasms.

Experiences and lessons

This case was initially considered to be a GIST. However, the strong immunostaining of S100 protein and GFAP suggested a peripheral nerve sheath tumor. The striking microcystic/reticular arrangement of the tumor cells helped to recognize the lesion as a special variant of schwannoma which was recently described.

Peer review

This article represents the first report of a microcystic/reticular schwannoma arising primarily in the vermiform appendix. Increased awareness of this special variant of schwannoma may help to avoid diagnostic pitfalls. More case reports are needed to further expand the clinical spectrum of the disease.

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Pericyst: The outermost layer of hydatid cyst

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Abstract

Hydatid disease, caused by the parasite *Echinococcus granulosus*, mostly affects the liver and the lungs with hydatid cysts which consist of three layers: (1) the outer pericyst; (2) the middle laminated membrane; and (3) the inner germinal layer. Pericyst, as the outermost layer of the hydatid cyst, is made by host cells encasing the hydatid cyst. An extremely close interaction exists between this host tissue and the parasite, and any degenerative changes of the pericyst would result in hydatid cyst degeneration or rupture. The pericyst plays an undeniably important role in the development and survival of the hydatid cyst.

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Key words: Hydatid disease; *Echinococcus granulosus*; Hydatid cyst; Pericyst; Layer

Core tip: Hydatid cysts consist of three layers: inner, outer and pericyst layers. An extremely close interaction exists between this host tissue and the parasite and any degenerative changes of the pericyst would result in hydatid cyst degeneration or rupture. The pericyst plays an undeniably important role in the development and survival of the hydatid cyst.

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TO THE EDITOR

Hydatid disease, caused by the parasite *Echinococcus granulosus*, is a major public health concern within the developing world. The primary hosts of the *Echinococcous granulosus* tapeworms are dogs. The eggs of the parasite are produced in the intestine of the primary hosts and passed *via* the feces. Later, eggs are ingested by intermediate hosts such as cattle[1]. Humans, as accidental hosts, might digest tapeworm eggs. In the upper gastrointestinal tract of the intermediate host, including humans (but not that of the primary host), the outer chitinous shells of hexacanth embryos become lysed, enabling the embryos to penetrate the mucosa of the duodenum and upper jejunum, enter the mesenteric venules and be carried in the portal stream to the liver. Theoretically, a few of the embryos can enter the lymphatics of the intestinal wall and bypass the liver through the cisterna chyli[2]. Numerous alternative routes have also been proposed which result in involvement of other organs including bypass of the hepatic sinusoidal venous system, a lymphatic route through the thoracic duct, the inhalation of air contaminated with *Echinococcus*, and direct infection *via* adjacent affected sites. Embryos are able to form cysts in almost every organ[3]. The diagnosis of a hydatid cyst is generally based on different imaging modalities and serology; however, serology is only useful for confirmation of the presumptive imaging diagnoses as it is nonspecific[4]. Non-invasive diagnostic approaches including ultrasonography are also used[5]. Although varied surgical methods have been introduced for the treatment of hydatid cysts, including enucleation, puncture, evacuation of cyst

contents, and cystotomy with or without capitonnage[6,7], complications including recurrence are frequent[8,9].

A hydatid cyst consists of three layers: (1) the outer pericyst; (2) the middle laminated membrane; and (3) the inner germinal layer. The pericyst, being the outermost zone of the hydatid cyst, and also known as the ectocyst or adventitial layer, consists almost entirely of host cells[10]. The initial endothelial cells encompassing the hydatid cyst can be ultimately transformed into a thin yet distinct capsule being replaced by fibroblasts, connective tissue and the leading parenchymal cells. The parasite can be completely encased by this host-originated adventitial zone forming the outermost layer of the hydatid cyst. Histopathological evaluations reveal fibrous strands enveloped by more closely-arranged cellular layers, while minute vascular spaces and fragments of small ductal channels of the host organ coexist[10]. Gradually, a blending of fibrous strands and compressed parenchymal cells with normal host tissue occurs so that no cleavage plane may be observed between the outermost portion of the layer and the surrounding normal host tissue. Osteopontin mainly distributes in the intra-pericystic layer of the hepatic hydatid cyst[11]. The diameters of pericysts differ depending on the host organ, but generally do not exceed a few millimeters. Two major mechanical or protective and nutritional functions have been hypothesized for the pericyst. The former, though difficult to assess, can be appreciated when considering the extreme fragility of the parasite. The latter becomes more apparent when considering that the total metabolic rate of the parasite, especially those of larger size, would require a constant nutritional supply, which is provided by the vascular channels within the pericyst. This suggests a very close interaction between the host tissue and the parasite, an association developing from the early stages of the follicle or vesicle to the fully-developed large cystic stage. Interestingly, degenerative changes of the pericyst herald an amorphous degeneration or spontaneous expulsion of a hydatid cyst. This can also be seen in the situation where non-vascular channels or ducts of the host organ, such as bile canaliculi in the liver, bronchial openings in the lungs or collecting tubules in the kidney, open up on the surface of the cyst initiating hydatid cyst rupture[6,8]. Different surgical approaches have been proposed for the treatment of hydatid disease.

In summary, hydatid cysts consist of three layers, *i.e.*, inner, outer and pericyst layers; this seems to have been overlooked by Lantiga *et al*[12] when describing the structure of the hydatid cyst in the clinical features of hydatid disease.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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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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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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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