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World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1362 experts in gastroenterology and hepatology from 67 countries.

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WJG 20th Anniversary Special Issues (4): Irritable bowel syndrome

Genetic polymorphism in pathogenesis of irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is a complex symptom-based disorder without established biomarkers or putative pathophysiology. IBS is a common functional gastrointestinal disorder which is defined as recurrent abdominal pain or discomfort that has at least two of the following symptoms for 3 d per month in the past 3 mo according to ROME Ⅲ: relief by defecation, onset associated with a change in stool frequency or onset with change in appearance or form of stool. Recent discoveries revealed genetic polymorphisms in specific cytokines and neuropeptides may possibly influence the frequencies and severity of symptoms, as well as the therapeutic responses in treating IBS patients. This review gives new insights on how genetic determinations influence in clinical manifestations, treatment responses and potential biomarkers of IBS.

Key words: Irritable bowel syndrome; Genetic polymorphism; Cytokines; Serotonin; Psychiatric distress; Endocannabinoids

Core tip: Irritable bowel syndrome (IBS) is a complex symptom-based disorder without established biomarkers or putative pathophysiology. This review gives new insights on how genetic determinations influence in clinical manifestations, treatment responses and potential biomarkers of IBS. Although a number of IBS- related genes have been identified, the majority of the identified genes required further validation as each of them may only contribute to the pathophysiology in 1%-5% in patients with functional gastrointestinal disorders.

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INTRODUCTION

Irritable bowel syndrome (IBS) is defined according to ROME Ⅲ criteria as recurrent abdominal pain or discomfort for at least 3 d per month during the previous months with two or more of the following characteristics: relief by defecation, onset associated with a change in the frequency of stools, onset associated with change in form or appearance of stools[1,2]. IBS is often subcategorized according to the predominant stool pattern reported by the patients. These subcategories include constipation-predominant IBS (IBS-C), diarrhea-predominant IBS (IBS-D), and so-called mixed stool pattern IBS (IBS-M) which involves both constipation and diarrhea.

The prevalence of IBS ranges from 4.7% to 19.1% in western countries, however the prevalence in eastern countries ranges from 3.7% to 15.7% according to ROME Ⅱ criteria[3]. Although the pathophysiology of IBS still remains unknown, there is growing evidence that genetic contributions, inflammatory activation, psychosocial factors may play important roles to the development of IBS.

Recent studies in genetic polymorphisms reported that cytokines and neuropeptides may be involved in etiology and clinical manifestations of IBS. This review summarizes the recent discoveries in association of genetic polymorphisms and their impacts on the symptom development and severity, pathogenesis as well as treatment responses to IBS.

GENE POLYMORPHISMS

As IBS is a complex symptom based disorder without single identified pathophysiology or biomarker, multiple mechanisms such as motility dysregulation, visceral hypersensitivity, immune activation, psychosocial factors and altered brain-gut axis has been proposed. Although a number of IBS- related genes have been identified, the majority of the identified genes required further validation. Besides, each of them may only contribute to the pathophysiology in 1%-5% in patients with functional gastrointestinal disorders[4]. This review will summarize how genetic determinations may possibly regulate the putative mechanisms mentioned.

Serotonergic system

Serotonin (5-HT) is one of the most abundant neurotransmitter molecules in the gastrointestinal tract. It is stored in the secretory granules of enterochromaffin (EC) cells in the enteric nervous system, and its release is believed to be responsible for eliciting appetite regulation[5], gut motility[6] and visceral sensitivity[7]. Abnormal levels and activities of 5-HT had been reported in functional gastrointestinal (GI) disorders such as functional dyspepsia (FD)[8] and IBS. Increased plasma 5-HT levels were found in female IBS-D patients[9-11] while decreased postprandial plasma serotonin levels have been reported in IBS-C patients[10]. Excessive 5-HT release in the bowel may lead to diarrhea, nausea and vomiting. Studies reported that single nucleotide polymorphisms (SNPs) in serotonin modulators showed significant associations with IBS. First, tryptophan hydroxylase (TPH) is the rate limiting enzyme in the biosynthesis of serotonin[12]. The two isoforms TPH1 and TPH2 showed associations to clinical manifestations in patients with IBS. TPH1 is located on chromosome 11p15.3-p14 and composed of 11 exons[13] and mainly expressed in gut and peripheral organs. TPH2 is located at chromosome 12q21.1 and composed of 11 exons[14] and mainly expression in CNS and peripheral neurons[15]. Homozygous for minor allele (GG) of rs4537731 in promoter region of TPH1 reported more severe diarrhea, bloating, and a trend of more watery stool compared to two genotype groups(AA and AG genotypes) in IBS patients[16]. Genotypes reported with a minor allele (GT and GG genotypes) of rs211105 in intron 3 of TPH1 also reported more severe diarrhea symptoms and trend of more watery stool. Homozygous for the minor allele (T) of TPH2 rs4570625 reported more days with both very hard and watery stools compared to other genotype groups (GG and GT genotypes) in the promoter region of TPH2[16].

Serotonin reuptake transporter (SERT) is a protein that removes serotonin from the sites of action and recycles serotonin back into presynaptic neurons. SERT is located on the chromosome 17q11.2-q1. Wang *et al*[17] showed that the homozygous genotype (L/L) in the promoter region of SERT (L variant bp-1440 to +22) can increase the mRNA and protein level expression of SERT promoter activity in the colonic mucosa. Yeo *et al*[18] found that a strong genotypic association was established between SERT promoter deletion/deletion genotype and female IBS-D patients. Fukudo *et al*[19] further reported SERT linked promoter region polymorphism with long (L, 528bp) and short (S, 484bp) forms showed different levels of brain activation after colorectal distention. This functional gene polymorphism may partially predict the individual effect of long-lasting neural processing from visceral organs. Camilleri *et al*[20] also reported that genetic polymorphisms at the SERT promoter influence response to a 5-HT3 antagonist in D-IBS patients. Kohen *et al*[21] reported that the carriers of rare G allele in polymorphism rs25531 of SERT linked promoter region showed threefold increase in odds ratio of IBS compared to healthy controls.

The 5-HT transporter gene linked polymorphic regions (5-HTTLPR), which is a 43bp insertion/ deletion polymorphism in the 5' flanking promoter region. It is 1.2 kb upstream of the transcription start site. Jarrett *et al*[22] showed the functional polymorphism(insertion or deletion of 44bp) in the 5-HTTLPR that was associated with depression and anxiety traits[23]. Furthermore, the 5-HTTLPR short allele has been found associated with increased visceral sensitivity in IBS[24]. Moreover, the L/L genotype was significantly associated with IBS, IBS-C and IBS-M patients in Korean population[25]. These may suggest that 5-HTTLPR might play a key role in IBS by modulation of SERT at transcriptional level.

Serotonin modulates visceral sensitivity by its action on 5-HT3 receptors. 5-HTR3 receptor A subunit (5-HTR3A), playing a key role in receptor formation, has been associated with depression and anxiety related trait. A functional polymorphism in 5-HTR3A subunit C-42C>T(rs1062613) was associated with more severe dyspeptic symptoms[26], increased anxiety, amygdala responsiveness and severity of IBS[27].

The 5-HT2A receptor subunit A (5-HTR2A) was believed to play a significant role in the genesis of various neuropsychiatric diseases. 5-HTR2A was reported to be responsible in regulating the perception of abdominal pain and smooth muscle contraction in gastrointestinal tract[28,29]. Markoutsaki *et al*[30] reported that the carriers of A allele of the -1438(G/A) polymorphism[31] and homozygote C allele of the 102 T/C polymorphisms in 5-HTR2A had higher risks of IBS[31]. Pata *et al*[31] showed that T/T genotype of 102 T/C polymorphism in 5-HTR2A may be associated with more severe pain in patient with IBS.

Cholecystokinin

Cholecystokinin (CCK) is released by endocrine I cells within the duodenal and jejunal mucosa for stimulating protein and fat digestion. It also served as a hunger suppressant[32]. Elevated plasma CCK level was reported to be associated with patients with post-infectious IBS. Plasma CCK level was correlated with postprandial dyspeptic

symptoms in these patients[33]. Study by Park *et al*[34] showed that polymorphism in CCK receptor intron 1 (779 T>C) was associated with constipation predominant IBS(IBS-C) and mixed IBS(IBS-M) in Korean population.

Catechol-O-methyltransferase

Catechol-*O*-methyltransferase (COMT) is involved in the inactivation of the catecholamine neurotransmitters. Altered COMT activities by different polymorphisms were related to chronic pain conditions such fibromyalgia[35] whereas the COMT Val158Met polymorphism had been associated to panic disorder[36] as well as IBS (Val/Val carriers showed a trend of smaller proportion of hard stools and higher occurrence of postprandial defecation)[37].

Voltage-gated sodium channel

Voltage-gated sodium channel (Na_v) was present in gastrointestinal smooth muscles. These missense mutations were found in tetrodotoxin-resistant sodium channel (SCN5A) in 13 out of 584 patients with irritable bowel syndrome. It was more prevalent in Diarrhea -predominant IBS patients. And these mutations showed disruption in Na_v 1.5 function with decreased peak currents and mechanosensitivity[4,38].

Guanine nucleotide binding protein beta polypeptide 3

Guanine nucleotide binding protein (G-protein) beta polypeptide 3 (GNB3) encodes the beta3 subunit of heterotrimeric G-protein. G-protein is responsible for various functions such as ion channel, motility and contraction. Lee *et al*[39] reported that a polymorphism in C825T has been associated with IBS-C patients in South Korea. Although no association was found between C825T with the overlapping of IBS and FD patients by Kim *et al*[40], an association was reported between dyspeptic symptoms and homozygous 825T allele of GNB3 protein in the H Pylori-negative Japanese population[41]. Oshima *et al*[42] also revealed epigastric pain syndrome(EPS) was correlated with homozygous 825T allele in GNB3 protein of patients with FD. Moreover, Saito *et al*[43] showed that an interaction was found between GNB3 825T allele and gastrointestinal infection of IBS in western population.

Endocannabinoid system

Endocannabinoids serve as synaptic circuit breakers and regulate multiple physiological and pathological conditions including nociception (pain sensation), appetite, lipid metabolism, gastrointestinal motility, cardiovascular modulation, motor activity, mood, and memory. Cannabinoids suppress behavioral responses to noxious stimulation and nociceptive processing through activation of cannabinoid CB receptor 1 (CNR1) and CB receptor 2 (CNR2) subtypes[44]. Wong *et al*[45] reported polymorphism of rs806378 (CT/TT genotype) in CB(1) receptor was associated with IBS patients having a modest delay in colonic transit. Camilleri *et al*[46] also showed that TT group had the fastest colonic transit at 24 and 48 h. Besides, there was a significant association of CNR1 in rs806378 with sensation rating of gas, but not pain sensation in various IBS subtypes. Park *et al*[47] and colleagues found a different distribution of allelic frequency of AAT repeats in the *CNR1* gene between healthy controls and IBS patients. They also reported a significant association of CNR1 >10/>10 genotype with IBS.

Psychiatric distress

Research has implicated that a combination of genetic and environmental risk factors (*e.g.*, Early life adversity, traumatic experiences) in the pathogenesis of mood disorders such as depression[48]. While strong association was established between psychological distress and functional gastrointestinal diseases[49], an established biopsychosocial model was suggested where early life stress may predispose HPA axis dysfunction and develop functional gastrointestinal symptoms[50]. The prevalence of depression and anxiety disorder was 37.1% and 31.4% respectively in Indian population with IBS[51]. Lee *et al*[52] also reported that IBS is also strongly associated with generalized anxiety disorder in Chinese population. Chronic widespread pain related to fibromyalgia and chronic fatigue is associated with IBS and major depressive disorder. Sato *et al*[53] showed that TT genotype of rs7209436 and rs242924 in Corticotrophin-releasing hormone was significantly more common in patients with IBS than in healthy controls. Corticotrophin-releasing hormone carries a potential risk for depression. These polymorphisms were also associated with bowel pattern in these IBS patients. Besides, polymorphisms in 5-HTTLPR, intron 2 (STin2 VTNR) of SERT were also correlated with depressive episodes and IBS[22].

Neuropeptide S (NPS) is a 20 amino acids peptide that selectively binds and activates an orphan G-protein coupled receptor, Neuropeptide S receptor 1 (NPSR1). It is expressed on the intestinal epithelium. This neuropeptide S system is involved in stress responses, anxiety, and nociception through selectively inhibiting the evoked release of serotonin and norepinephrine the frontal cortex, by acting directly on serotonin and norepinephrine nerve terminals[54]. NPSR1 polymorphisms were reported to be associated with colonic transit rate (rs2609234, rs6972158 and rs1379928) and visceral pain(rs1379928)[55].

Cytokines

It has become increasingly clear that low-grade inflammation is implicated in the pathophysiology of IBS with subtle changes in pro-inflammatory or anti-inflammatory cytokines in blood or GI mucosa[56,57]. Studies reported significant associations between functional polymorphisms in these genes among IBS patients. Tumour necrosis factor alpha (TNFα) is a cytokine which involved in stimulating systemic inflammation and it is implicated in various diseases such as cancer, depression and inflammatory bowel disease. Although polymorphism in TNFα(-308 G/A) showed no difference in frequencies between Indian IBS patients and healthy volunteers[58], Barkhordari *et al*[59] showed that polymorphism of TNFα at position -308 and -238 were also

significantly higher in IBS patients. TNFSF15 is a member of the TNF (ligand) superfamily which codes for TL1A. It is expressed primarily in macrophage, T cells, and immune cells that are exposed to pro-inflammatory stimuli or microbes. TNFSF15 involves in the defense against pathogens and homeostatic interactions with commensal bacteria in the gut. Study by Zucchelli *et al*[60] showed the Crohn's disease risk allele rs4263839 G in TNFSF15 gene was significantly associated with increased risk of IBS and particularly in IBS-C patients. TNFRSF17 is expressed in mature B lymphocytes and development of B cells. Besides its involvement in inflammatory bowel disease (IBD), SNPs at position -1729G, -2445 and -2493 showed significantly distinct frequencies in patients with lower functional gastrointestinal disorders compared to healthy controls[61].

Interleukins are important modulators in inflammatory responses, they play a vital role in intestinal inflammation. Pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 has been up-regulated in IBS patients[56]. Although Camilleri *et al*[62] showed no difference in gene polymorphisms of IL-6 was found between subtypes of IBS patients and healthy individuals in American population, IL-6 G allele at position -174 showed higher frequencies in Iranian IBS patients[59]. Moreover, there was significant difference in frequencies shown in IL-8 G allele at position +396 and C allele +781 between IBS patients and healthy controls. Besides, the combinations of IL-8 ATCC haplotypes (at positions -251, +396, +781 and +1633) reported significant association with susceptibility to development of IBS[63]. In Mexican population, IL-8 T allele at position +781 was significantly overexpressed in patients with IBS and IL-8 G allele at position +396 was also associated with IBS[64].

Anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF) β1 also play an important role in the regulation of immune and inflammatory responses. Although no significant difference was found in colonic expression of TGFβ1 in IBS patients[65], Romero-Valdovinos *et al*[63] showed that IL-10 A allele at position 1082 were significantly increased in IBS Mexican patients. IL-10 can inhibit pro-inflammatory cytokines such as tumour necrosis factor beta (TNF-β). IL-10 ACC haplotypes (at positions -1082, -819 and -592) were also associated with development of IBS. IL-10 C allele at position -592 also showed association with higher risk in developing IBS in Mexican population[64].

CONCLUSION

IBS is a complex functional disorder that involves multiple interactions of genetic inheritance, environmental and psychosocial factors. This review summarized the recent discoveries on how genetics may influence on the symptoms severity and subtypes of IBS through modulation of gastrointestinal functions such as gut motility, immune activation and visceral sensation. Further studies are necessary to understand the mechanisms on how genetics may determine the clinical manifestations and therapeutic responses to subset of IBS patients. Besides, biomarker discovery for this complex heterogeneous disorder remains a big challenge. Future studies should be required to search for candidate genes with combinations of gene expression profiling for target treatment and diagnosis for specific subsets of IBS patients.

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TOPIC HIGHLIGHT

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

Toll-like receptor signaling in colorectal cancer: Carcinogenesis to cancer therapy

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Abstract

Toll-like receptors (TLRs) are germ line encoded innate immune sensors that recognize conserved microbial structures and host alarmins, and signal expression of major histocompatibility complex proteins, costimulatory molecules, and inflammatory mediators by macrophages, neutrophils, dendritic cells, and other cell types. These protein receptors are characterized by their ability to respond to invading pathogens promptly by recognizing particular TLR ligands, including flagellin and lipopolysaccharide of bacteria, nucleic acids derived from viruses, and zymosan of fungi. There are 2 major TLR pathways; one is mediated by myeloid differentiation factor 88 (MYD88) adaptor proteins, and the other is independent of MYD88. The MYD88-dependent pathway involves early-phase activation of nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB1) and all the TLRs, except TLR3, have been shown to activate this pathway. TLR3 and TLR4 act *via* MYD88-independent pathways with delayed activation of NF-κB signaling. TLRs play a vital role in activating immune responses. TLRs have been shown to mediate inflammatory responses and maintain epithelial barrier homeostasis, and are highly likely to be involved in the activation of a number of pathways following cancer therapy. Colorectal cancer (CRC) is one of the most common cancers, and accounts for almost half a million deaths annually worldwide. Inflammation is considered a risk factor for many common malignancies including cancers of the colorectum. The key molecules involved in inflammation-driven carcinogenesis include TLRs. As sensors of cell death and tissue remodeling, TLRs may have a universal role in cancer; stimulation of TLRs to activate the innate immune system has been a legitimate therapeutic strategy for some years. TLRs 3/4/7/8/9 are all validated targets for cancer therapy, and a number of companies are developing agonists and vaccine adjuvants. On the other hand, antagonists may favor inhibition of signaling responsible for autoimmune responses. In this paper, we review TLR signaling in CRC from carcinogenesis to cancer therapy.

Key words: Toll-like receptor; Colorectal cancer; Carcinogenesis; Prognosis; Cancer therapy

Core tip: Toll-like receptors (TLRs) are innate immune

sensors which can recognize inflammatory mediators. TLRs have been shown to mediate inflammatory response and maintain epithelial barrier homeostasis. Inflammation is a risk factor for many cancers including colorectal cancer (CRC). The key molecules involved in inflammation-driven carcinogenesis include TLRs. In this paper, we reviewed TLR signaling in CRC from carcinogenesis to cancer therapy.

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INTRODUCTION

Toll-like receptor biology

Toll-like receptors (TLRs) are a family of evolutionally conserved pattern recognition receptors (PRRs)[1-3]. TLRs are included in the type I transmembrane glycoprotein receptor family with N-terminal ligand-recognition, transmembrane, and intracellular C-terminal signaling domains[4]. Currently, 13 TLRs have been identified in humans and mice, and equivalent forms of many of these have been found in other mammalian species[5]. TLRs recognize a wide range of microbial moieties, and engagement by their respective ligand(s) triggers activation of intracellular signaling cascades leading to the induction of genes involved in antimicrobial host defence, such as those encoding proinflammatory cytokines and chemokines[6,7].

TLR signaling has been investigated extensively in recent years. There are two important TLR pathways: one is dependent on myeloid differentiation factor 88 (MYD88) adaptor proteins and the other is independent of MYD88. All TLRs commonly use MYD88 as the downstream adapter protein except TLR3. After activation with their individual ligands, TLRs recruit MYD88, leading to subsequent activation of downstream factors, including nuclear factor κB (NF-κB), mitogen-associated protein kinase (MAPK), and interferon (IFN) regulatory factors[8,9]. TLR signaling activates transcription factors, and generates cytokines as well as chemokines via intracellular pathways (Figure 1). TLR2 and TLR4 combine with their respective ligands to form dimeric complexes. The configuration is then changed and 5 specific adapters within cells are recruited, including MYD88, TIR domain-containing adaptor protein (TIRAP)/MYD88 adaptor-like (Mal), TIR domain-containing adaptor-inducing IFNβ (TRIF), TRIF-related adaptor molecule (TRAM), as well as sterile α and armadillo motif-containing protein (SARM)[4]. Immune cell expressing TLRs play important roles in immune responses against invading pathogens. TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) expressed on a wide array of microbes, as well as danger-associated molecular patterns (DAMPs) released from stressed or dying cells[10].

TLR in disease and cancer

TLRs play a major role in microbe-host interactions and innate immunity[11]. TLRs are very important in early innate immune defense mechanisms by activating canonical and non-canonical pathways of inflammation. Because TLRs are primary sensors of PAMPs, DAMPs, and stress signals associated with allergen exposure, genetic variations in the TLR genes may influence the incidence, severity, and outcome of allergic diseases[2]. Several single-nucleotide polymorphisms within the TLR genes are associated with altered susceptibility to infectious, allergic, and inflammatory diseases as well as cancers[2].

More and more evidence suggests that malfunction of TLR signaling contributes significantly to the development of autoimmune connective tissue diseases[3], tuberculosis[9], severe acute pancreatitis[12], necrotizing enterocolitis[13], atherosclerosis[14], alcohol-induced liver disease and non-alcoholic steatohepatitis[15]. TLR signaling plays a role in regulating injury responses of chronically injured precancerous organs and promoting malignant cell survival[16]. The TLR/MYD88 pathway is essential for microbiota-induced development of colitis-associated cancer, and it was demonstrated that the severity of chronic colitis directly correlates with colorectal tumor development and that bacterial-induced inflammation drives progression from adenoma to invasive carcinoma[17]. TLRs and MYD88 signaling have been shown to be associated with hepatic inflammation and hepatomitogen expression which is important for hepatocarcinogenesis, suggesting that a better understanding of TLR signaling pathways may help to clarify the mechanisms of tumorigenesis, and provide new therapeutic targets for hepatocellular carcinoma[15]. Researchers have found that TLR9 initiates a cascade of immune responses: expression of TLR9 promotes angiogenesis and cancer progression, and reduces survival, so an understanding of how TLR9 boosts angiogenesis may help refine the development of anticancer agent[18,19]. Table 1 showed TLR functions in disease and cancer.

TLR in therapy

As the evidence for the involvement of TLRs in multiple immune diseases has increased, more and more research has shown that TLRs could be a therapeutic target for inflammatory diseases. TLR2 could be a useful therapeutic target for the development of antagonists given the range of diseases that are associated with this receptor[20]. The humanized version of OPN-305 entered phase I clinical trials for the treatment of inflammatory autoimmune diseases[20-22]. Small synthetic compounds, acting as TLR3 agonists and/or TLR2/TLR4, TLR7/9 and MYD88 antagonists may favor the inhibition of signaling responsible for autoimmune responses in multiple sclerosis and experimental autoimmune encephalitis[20].

Various TLR agonists have been considered for multiple clinical applications, including cancer immunotherapy, and the TLR7 agonist imiquimod is approved for topical

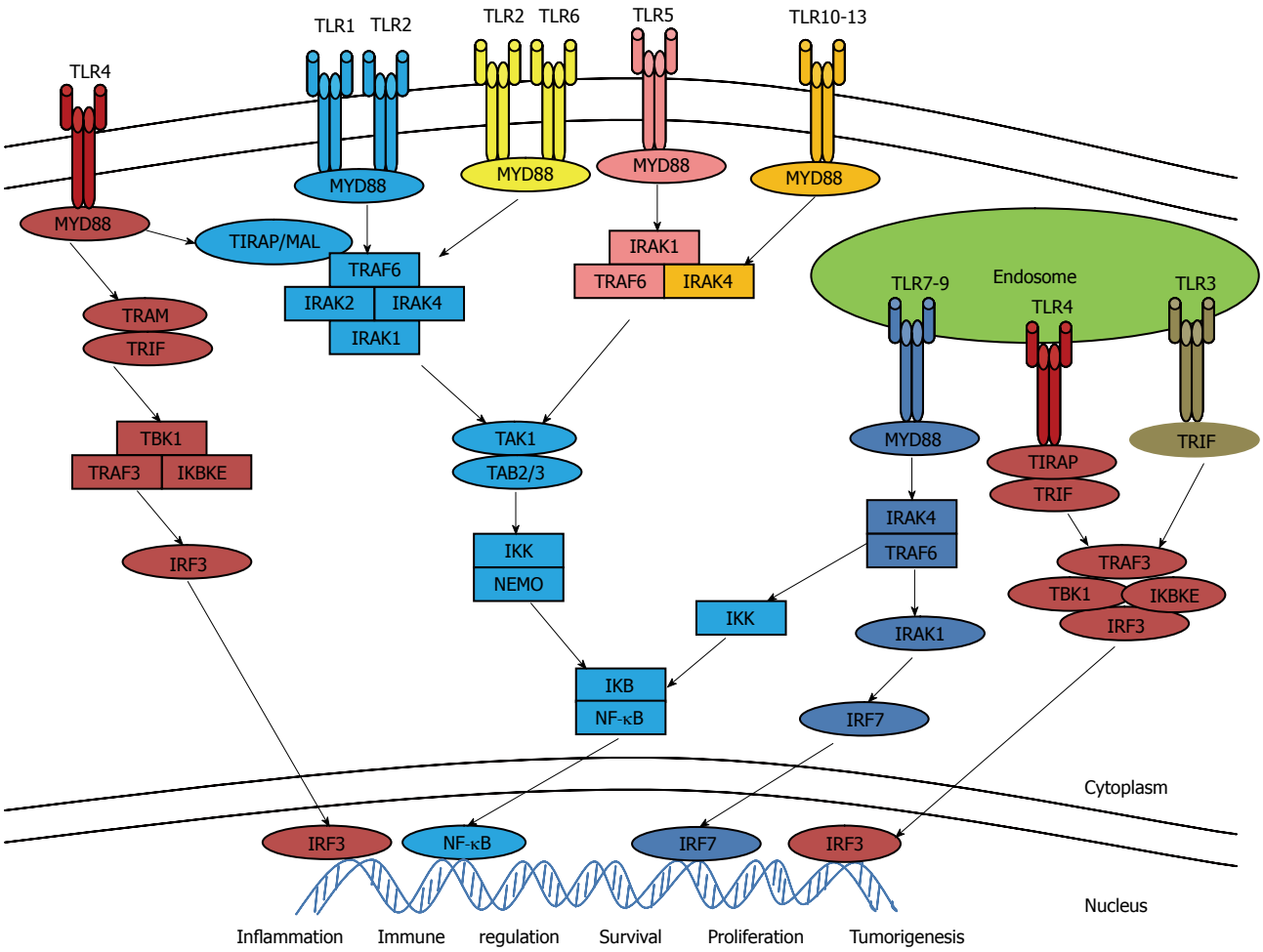

Figure 1 Toll-like receptor signaling pathways. There are 2 major toll-like receptor (TLR) pathways: One is mediated by myeloid differentiation factor 88 (MYD88) adaptor proteins, and the other is independent of MYD88. With the exception of TLR3, all other TLRs commonly use MYD88 as the downstream adapter protein. Activated TLRs recruit MYD88, leading to subsequent activation of the downstream targets. TLR2 and TLR4 combine with their respective ligands to form dimeric complexes and change their configuration, and then recruit specific adapters within cells, including MYD88, TIR domain-containing adaptor protein (TIRAP)/MYD88 adaptor-like (Mal), TIR domain-containing adaptor-inducing interferon (IFN)-β (TRIF) and TRIF-related adaptor molecule (TRAM). The subsequent activation of the IKK complex, consisting of TBK1/TRAF3/IKKε, NEMO/IKBKE, induces phosphorylation of IKBKE, leading to the activation of transcription factors. TLRs bind bacterial and viral PAMPs, leading to activation of proinflammatory and anti-viral signaling pathways including NF-κB1 and IFN regulatory factor-3 (IRF3) and 7 (IRF7), then activation of transcription factors and production of inflammatory cytokines, leading to inflammation, immune regulation, survival, proliferation and tumorigenesis.

Table 1 Toll-like receptor functions in disease and cancer

TLR	Disease	Function
TLRs	Autoimmune disease	Microbe-host interactions and innate immunity
TLRs	Infectious disease	Activating canonical and non-canonical pathways of inflammation
TLRs	Cancer (CRC, HCC, *etc*)	Carcinogenesis
TLRs	Allergic diseases	Primary sensors of PAMPs, DAMPs and stress signals associated with allergen exposure
TLRs	Tuberculosis	Recognition of Mycobacterium tuberculosis
TLRs	Systemic inflammatory response syndrome	Development of syndrome
TLR2/TLR4	Atherosclerosis	Development of disease
TLR4	NEC	Development of intestinal barrier failure
TLR4	Alcohol-induced liver injury	Activation of Kupffer cells
TLR9	NASH	Development of disease
TLR9	Cancer	Angiogenesis

TLR: Toll-like receptor; CRC: Colorectal cancer; HCC: Hepatocellular carcinoma; PAMPs: Pathogen-associated molecular patterns; DAMPs: Danger associated molecular patterns; NEC: Necrotizing enterocolitis; NASH: Non-alcoholic steatohepatitis.

therapy of basal cell carcinoma[23]. Most TLR-targeted therapeutics are intercellular nucleic acid-derived immunoregulatory sequences. such as TLR3, TLR7, TLR8, and TLR9. Agents can also target cell surface TLRs, including TLR2 and TLR4. These therapeutics may be used in oncology, immune disease, and infectious disease[1].

Activation of the TLR4 pathway may cause chronic inflammation and increase production of reactive oxygen and nitrogen species (ROS/RNS), leading to oxidative and nitrosative stress and TLR-related diseases. This implies that drugs or substances that modify these pathways may prevent or improve TLR-related diseases, for example, anti-lipopolysaccharide (LPS) strategies, aim to neutralize LPS and TLR4/MYD88 antagonists, including eritoran, CyP, EM-163, epigallocatechin-3-gallate, 6-shogaol, cinnamon extract, N-acetylcysteine, melatonin, and molecular hydrogen[24]. Rajput *et al*[25] correlated TLR4 expression with resistance to paclitaxel in either depleted or overexpressed TLR4 protein breast cancer cell lines and found that paclitaxel not only killed tumor cells but also enhanced their survival by activating the TLR4 pathway, suggesting that blocking TLR4 could significantly improve the response to paclitaxel therapy. TLR4 is critical for the airway inflammatory response, and agents targeting TLRs are being actively pursued as novel therapies for the treatment of airway diseases such as asthma[26]. Synthetic oligodeoxynucleotide-expressing CpG motifs (CpG-ODN) are TLR9 agonists that can enhance the antitumor activity of DNA-damaging chemotherapy and radiation therapy in preclinical mouse models, and findings provide evidence that the tumor microenvironment can sensitize cancer cells to DNA-damaging chemotherapy, thereby expanding the benefits of CpG-ODN therapy beyond induction of a strong immune response[27]. TLR9 agonists can exert antitumor effects by blocking angiogenesis; it is likely that TLR-induced IFNs play an important role as IFNα is well known to suppress tumor angiogenesis[16].

TLR IN COLORECTAL CANCER CARCINOGENESIS

General introduction

Colorectal cancer (CRC) is the fourth leading cause of cancer-related death in the world and the third leading cause in the United States[28]. Initiation and progression of malignancies is the result of a series of complex processes that depend upon multiple interactive factors[29]. There are 3 distinct molecular mutagenic pathways, including chromosomal, microsatellite instability, and epigenetic pathway in colon carcinogenesis[11,22].

Inflammation is considered a risk factor for many common malignancies including CRC[29,30]. The key molecules involved in inflammation-driven carcinogenesis include TLRs, NF-κB signaling, pro- and anti-inflammatory cytokines, growth factors, kinase tumor suppressor proteins, cyclooxygenases, and nitric oxide synthases[31]. Pimentel-Nunes *et al*[32] found persistently positive TLR expression and lower expression of TLR inhibitors associated with higher TLR protein levels throughout the spectrum of lesions of colon carcinogenesis[22]. TLR3 may indicate the tendency of normal tissue to form adenoma or CRC[17].

Each receptor in CRC carcinogenesis (in vivo and in vitro)

TLR2: TLR2 is encoded by a DNA sequence that codes 784 amino acids[9]. This type I transmembrane receptor is composed of an extracellular leucine-rich domain, a single transmembrane domain, and a cytoplasmic domain[9]. Colon carcinogenesis is associated with increased expression levels of TLR2 and TLR4. Functional TLR2 and TLR4 polymorphisms significantly alter the risk of CRC. Smoking and obesity may influence the risk of CRC along with these genetic profiles[33]. TLR2 is unique in its requirement to form heterodimers with TLR1 or TLR6 for the initiation of signaling and cellular activation[11]. Tumor cells from TLR2 knockout mice showed less cell death and suppressed senescence[16]. Nihon-Yanagi *et al*[34] suggested that TLR2 activation may also be involved in sporadic colon carcinogenesis in humans.

In CRC , the role of TLR2 is still controversial. One study showed that there were no differences between wild-type and TLR2-deficient mice in CRC[16,35]. However, another study showed increased tumor development and higher interleukin (IL) 6, IL17A and phospho-signal transducer and activator of transcription 3 (STAT3) levels in CRC in TLR2-deficient mice[16,36]. In colitis, TLR2 plays a protective role against the development of colitis-associated cancer[36]. TLR2 plays a key role in Gram-positive bacterial, mycobacterial, fungal, and spirochetal cell wall component recognition, while TLR4 seems to be a key receptor of the Gram-negative component LPS; both TLR2 and TLR4 in cancer patients are implicated in carcinogenesis and antitumor treatment; the lower stress response in laparoscopic colectomy *vs* open colectomy provides an impetus to investigate the long-term results of laparoscopic colectomy *vs* open colectomy for CRC[37]. Some papers also showed that TLR2 and TLR4 were both associated with survival after diagnosis of colon cancer, but not rectal cancer[38].

TLR4: TLR4 is composed of 839 amino acids. It is activated by bacterial LPS as well as lipotechoic acid[9]. TLR4 is expressed on human colon cancer cells and is functionally active. It is important in promoting immune escape of human colon cancer cells by inducing immunosuppressive factors as well as apoptosis resistance[31]. The TLR4 signaling pathway has oncogenic effects both *in vitro* and *in vivo*. The increased individual expression of TLR4 and IL6 is a common feature of CRCs and is associated with poor prognosis[39-41]. To demonstrate the role of TLR4 signaling in colon tumorigenesis, Wang *et al*[39] examined the expression of TLR4 and MYD88 in CRC, and suggested that high expression of TLR4 and MYD88 is associated with liver metastasis, and is an independent predictor of poor prognosis in patients with CRC. Their findings also sug-

gest that TLR4/MYD88 signaling contributes to CRC tumorigenesis not only in colitis-associated cancer but also in sporadic CRC[39]. Other studies also showed that TLR4 signaling activates NF-κB through the MYD88 pathway, leading to transcription of pro-inflammatory cytokines as well as many important components of the inflammatory response[42].

TLR4 is overexpressed in mouse and human inflammation-associated CRC, and TLR4-deficient mice are strongly protected against colon carcinogenesis, suggesting that TLR expression on tumor cells promotes tumor progression directly or indirectly[8]. TLR4 expression by stromal fibroblasts is associated with poor prognosis in CRC[43]. The TLR4 variant D299G induces neoplastic progression in Caco-2 intestinal cells and is associated with advanced human colon cancer, implying a novel link between colonic carcinogenesis and aberrant innate immunity[44]. Single TLR4, LY96 (MD-2), and CXC chemokine receptor 7 (CXCR7) expression levels are significantly correlated with human CRC TNM stage, advanced histological grade, tumor size, and lymph node metastasis; furthermore, concomitant expression of TLR4, LY96 and CXCR7 has been shown to be associated with increased potential for carcinoma growth and metastasis in human CRC[29]. Cammarota *et al*[45] found that adenocarcinoma patients (pT1-4) with higher TLR4 expression in the stromal compartment had a significantly increased risk of disease progression, and high TLR4 expression in the tumor microenvironment represents a possible marker of disease progression in colon cancer. Nox enzymes are major sources of endogenous ROS generation in response to inflammatory mediators, including cytokines, growth factors, and hypoxic conditions, all of which are elevated in response to surgical trauma[42,46,47]. It was shown that the LPS-Nox1 redox signaling axis plays a crucial role in facilitation of colon cancer cell adhesion, thus increasing the potential for colon cancer cell metastasis. Nox1 may represent a valuable target to prevent colon cancer metastasis[42].

TLR9: TLR9 recognizes unmethylated CpG motifs in bacterial DNA[9]. TLR9 is expressed mainly in intracellular vesicles such as the endoplasmic reticulum, lysosomes, endosomes and endolysosomes, where they recognize microbial nucleic acids[1]. TLR9 recognizes DNA derived from both DNA bacteria and viruses[1,48]. Several studies have shown that TLR9 engagement on CD4 T cells can enhance their survival and therefore, could potentiate antitumor responses by prolonging T cell survival[10,49]. The role of TLR9 signaling in colonic carcinogenesis remains unclear. It was recently reported that oligodeoxynucleotides targeting TLR9 have opposite effects in modulating DNA repair genes in tumor cells *vs* immune cells, and enhance the biologic effects of chemotherapy. TLR9 expression was decreased in hyperplastic and villous polyps from patients who developed CRC, suggesting a possible protective role of TLR9 expression against malignant transformation in the colorectal mucosa[50]. Table 2 and Figure 2 show the TLRs involved in CRC.

TLR IN CRC PROGNOSIS

It was reported that high expression of the TLR4/ MYD88 signal was correlated with poor prognosis of CRC[51]. In the tumor microenvironment, high TLR4 expression represents a possible marker of disease progression in colon cancer[45]. TLR4 expression in stromal fibroblasts is associated with poor prognosis in CRC, therefore, TLR4 expression in fibroblasts could be a useful prognostic marker in CRC[43]. It has been documented that the deregulated activation of STAT3 and NF-κB is a common feature of gastrointestinal cancers and invariably correlates with poor prognosis; NF-κB and STAT3 are key downstream signal transducers of the TLR families and IL-6 cytokine, respectively; the molecular mechanisms are associated with cross-talk between the IL-6 cytokine family/STAT3 signaling network and the TLR family/NF-κB signaling network, and there is potential benefit in their therapeutic targeting in colorectal and gastric cancers[7]. Genetic variations in TLR2, TLR3 and TLR4 may influence colon cancer development as well as survival after diagnosis with colon cancer[38]. Persistent TLR-specific activation of NF-κB in CRC and particularly in tumor-initiating cells may sustain further tumor growth and progression through perpetuation of signaling from inflammatory and tissue repair mechanisms, with consequent self-renewal of pluripotent tumor cells. TLR7 and TLR8 expression on PROM1 $(CD133)^+$ cells in CRC may play a specific role in tumorigenesis and tumor progression[52].

TLR IN CRC THERAPY

Agonists

TLR agonists play a fundamental role in activating innate and adaptive immune responses[10]. TLR agonists are currently under investigation as vaccine adjuvants in anticancer therapies for their ability to activate immune cells and promote inflammation[10]. A growing body of evidence indicates that TLRs are expressed or can be induced on various cell types, including T cells and tumor cells[8,10].

Current available synthetic TLR2 ligands are based on cell wall constituents of (potential) pathogens, and adjuvant research could possibly benefit from elucidating the variations in the LPS make-up of probiotic strains. With regard to the indispensable role of pattern recognition receptors (PRRs) in facilitating microbe-induced TLR2 function, determination of specific PRRs involved in the recognition of probiotic strains would aid research on the mechanism of action of probiotics. In addition, because microbial manipulation of PRR-TLR crosstalk is used by pathogens to subvert appropriate immune responses, determination of the specific PRRs involved could lead to new therapeutic approaches[11]. The TLR2/4 agonists S100A9 and HMGB1 have been touted as potential biomarkers for CRC, as they are upregulated significantly in

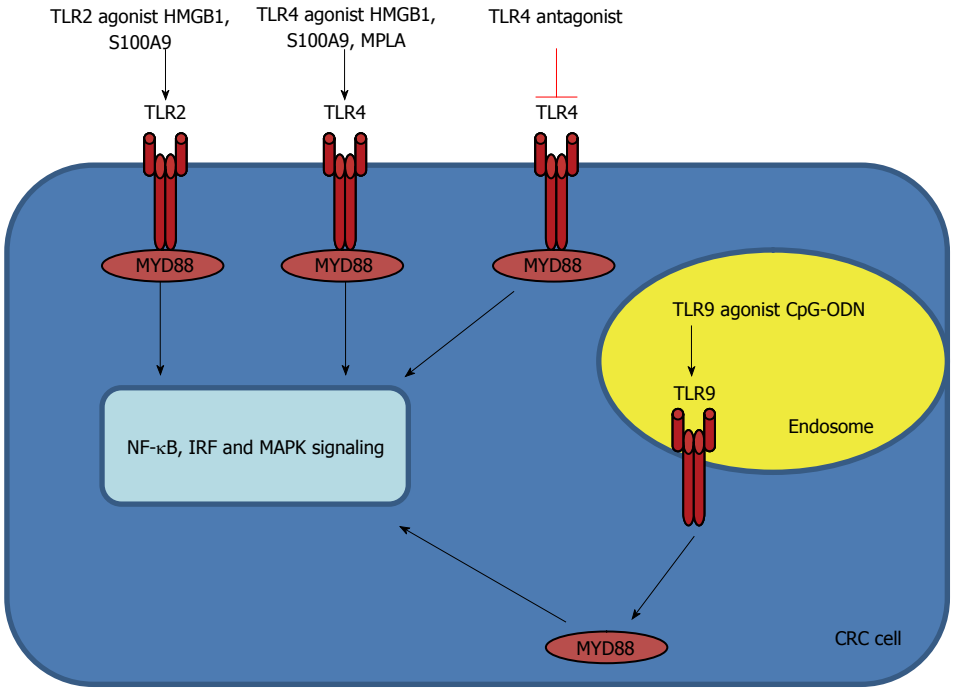

Figure 2 Toll-like receptors in colorectal cancer and therapeutics. Toll-like receptor (TLR) agonists play a fundamental role in activating innate and adaptive immune responses. The TLR2 and TLR4 agonists HMGB1 and S100A9 have been proposed as potential biomarkers for colorectal cancer (CRC). The TLR4 agonist monophosphoryl lipid (MPL) A is approved for use in several vaccines as an adjuvant. TLR9 agonist, commonly referred to as CpG-ODN, has been added to the arsenal of anti-cancer drugs as monotherapy or in combination with chemotherapy, radiotherapy and other immunotherapeutic approaches. Activated TLR2, TLR4 and TLR9 recruit MYD88, with activation of NF-κB, IRF, and MAPK signaling, leads to inflammation, immune regulation, survival, proliferation and tumorigenesis. MAPK: Mitogen-associated protein kinase; IRF: Interferon regulatory factor; NF-κB: Nuclear factor (NF)-κB.

Table 2 Toll-like receptors role in colorectal cancer

TLR	Carcinogenesis	Prognosis	Treatment
TLR2	Controversial role in mouse model; protective against development of CRC in colitis	Associated with survival after diagnosis of colon cancer	HMGB1, S100A9
TLR4	Oncogenic effects *in vitro* and *in vivo*	Poor progression	HMGB1, S100A9, MPLA
TLR9	Remain unclear; possible protection against malignant transformation in colorectal mucosa		CpG-ODN

CRC: Colorectal cancer; TLR: Toll Like receptor.

CRC and have been shown to be regulated by STAT3, which is hyperactivated in approximately 90% of colorectal tumor biopsies[41,53,54]. The TLR4 agonist monophosphoryl lipid A is approved for use in several vaccines as an adjuvant[1,51].

Rosa *et al*[55] established a *KRAS* mutated CRC model and showed that an immunomodulatory oligonucleotide sequence in combination with cetuximab had an antitumor effect. This is probably based on the alteration of MAPK phosphorylation, resulting in structural and functional changes in the relationship between epidermal growth factor receptor (EGFR) and TLR9[50,55]. Mutation of the *KRAS* gene has a critical role in colon cancer and may cause resistance to anti-EGFR therapy, which is the reason why panitumumab and cetuximab therapy do not show a positive effect on the control of proliferation and metastasis in *KRAS*-mutated colon cancer; this kind of biological therapy could only be useful in the case of patients carrying the wild-type *KRAS* gene; estrogen receptors may take part in colorectal carcinogenesis, and interaction between TLR9 and estrogen receptors may have further therapeutic importance in CRC, and TLR9 agonist therapy has been tested clinically on the colon[50]. The TLR9 agonist, which is commonly referred to as CpG-ODN, has been added to the arsenal of anti-cancer drugs as monotherapy, or in combination with chemotherapy, radiotherapy, and other immunotherapeutic approaches, as they increase antigen presentation and boost anti-tumor B and T cell responses[56]. TLR9 agonists were reported to show TP53-independent activity within human CRC cells, inhibit their proliferation, promote apoptosis, and improve anti-cancer effects of radiotherapy and chemotherapy[17]. One therapeutic advantage of the use of TLR9 agonists in this tumor model could be to sensitize tumors to the toxic effects of radiation treatment[10,57]. Combined administration of a TLR9 agonist and an

Table 3 Summary of therapy in colorectal cancer

Compound	Target (agonist)	Indications	Drug class or trade	Clinical phase
BCG[10,71,72]	TLR2/4	CRC	Synthetic ssRNA	Phase Ⅰ
MPL[10,72]	TLR4	CRC	Synthetic ssRNA	Phase Ⅰ
CBLB502[1,23,71]	TLR5	Colon cancer	Flagellin	Phase Ⅰ
Imiquimod (Aldara)[10,71,72]	TLR7	CRC	Small molecule ssRNA	Phase Ⅰ / Ⅱ / Ⅲ
IMO2055[1,10]	TLR9	CRC	CpG oligonucleotide	Phase Ⅰ / Ⅱ
MGN1703[16]	TLR9	CRC	dSLIM	Phase Ⅱ

IL-10 antagonist is one of the candidates for cancer treatment[8]. Recently, it was shown that TLR ligands may be critical for dendritic cell (DC) activation, and combined TLR activation can lead to better DC maturation status, and also induce more effective antitumor immune responses against colon cancer, showing that it may be a potential strategy to develop more powerful DC cancer vaccines[58].

It was reported that specific small molecule inhibitors of phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PIK3CA) reduce immunosuppression to increase the proinflammatory effects of TLR ligands that support antitumor immunity. Multiple strategies to inhibit PIK3CA in DC led to IL-10 and transforming growth factor-β1 suppression but did affect IL12 or IL1B induction by the TLR5 ligand flagellin[59].

Antagonist

TLR4 plays an important role in innate immunity as the first line of host defense. Most human cells express high levels of TLR antagonist proteins and a low level of TLR4. Tumor progression involves TLR4-mediated irregular and uninhibited production of proinflammatory cytokines, immunosuppressive cytokines as well as chemokines; suggesting that the discovery of TLR4 antagonists may be an ideal strategy to treat tumors. TLR4 antagonists were found to pose a risk of compromising host immunity in other studies, so that it is a scientific dilemma whether a TLR4 agonist or antagonist should be targeted as treatment for cancer[60]. The TLR4/LY96 antagonist antibody inhibited colitis-associated neoplasia in a mouse model, and it was shown that TLR regulation can affect the outcome of both acute colitis and its consequences, *i.e.*, cancer. Targeting TLR4 and other TLR's may ultimately play a role in prevention or treatment of colitis-associated cancer[61].

TLR IN CLINICAL TRIALS

The developmental process for TLR-targeting products in cancer has not been altogether straightforward, and two of the earliest TLR pioneers have had disappointing results. However, there are a number of promising second-generation products currently in development, and targeting of TLR9 for metastatic CRC in clinical phase Ⅱ / Ⅲ trials is being performed by Mologen company[45]. Various TLR agonists are currently under investigation in clinical trials for their ability to orchestrate antitumor immunity[10]. Pollinex Quattro (Allergy Therapeutics Ltd., Worthing, UK) is a vaccine that contains a monophosphoryl lipid adjuvant to stimulate TLR4, combined with ragweed pollen extract for the treatment of seasonal allergic rhinitis[1,62]. Following positive results in phase Ⅲ trials, Allergy Therapeutics have submitted Pollinex Quattro for regulatory approval in Europe[1]. TLR9 is a key determinant of the innate immune responses in both sterile and infectious injury. Specific TLR9 antagonism reduces tissue damage in a wide range of pathologies, and has been delivered by modification of nucleic acids, a recognized ligand for TLR9, and a novel small-molecule enantiomeric analogue of traditional morphinans which has specific TLR9 antagonist properties and reduces sterile inflammation-induced organ damage[52]. Some of the TLR-based therapeutics under evaluation in CRC are shown in Figure 2 and Table 3.

CONCLUSION

TLRs are very interesting receptors and are highly important in the field of adjuvant, pathogen, and probiotic research. TLRs constitute a link between adaptive (specific) and innate (non-specific) immunity, contributing to the capacity of our immune system to efficiently combat pathogens. They also enable immune cells to discriminate between self and nonself antigens[17]. TLRs are connected to the cell signaling machinery *via* intracellular adaptor molecules, and stimulation of the TLR/IL1R signaling pathway activates the major inflammatory transcription factor NF-κB1 by allowing its nuclear translocation[63]. Predictably, MYD88 was shown to play a role in tumorigenesis *via* TLR and IL1 proinflammatory mechanisms[63-65]. TLR-mediated signaling can promote tumor growth, and using a TLR agonist or antagonist in combination with an antigen isolated from tumors may increase the effect of vaccination and evoke specific innate immunity against a tumor[6]. TLR stimulation results in NF-κB1 activation, a key modulator in driving inflammation to cancer and mitogen-activated protein kinases that have been shown to recruit mitotic and prostaglandin endoperoxide synthase 2 (PTGS2)-induced pathways in carcinogenesis[66].

CRC is a major cause of cancer-associated morbidity and mortality worldwide, and is the third most common cancer in men and women; in addition, CRC is the third leading cause of cancer-related deaths, and the incidence of this disease is increasing[67,68]. The role of TLRs in

CRC pathology has not been fully elucidated. Bacterial infection stimulates the TLR/MYD88 pathway in tumor tissues, which leads to the induction of PTGS2 in stromal cells, including macrophages, and induction of the PTGS2/PGE(2) pathway in tumor stroma is important for the development and maintenance of an inflammatory microenvironment in gastrointestinal tumors[69]. Persistent TLR-specific activation of NF-κB in CRC, and particularly in tumor-initiating cells, may thus sustain further tumor growth and progression through perpetuation of signaling in inflammatory and tissue repair mechanisms, with consequent self-renewal of pluripotent tumor cells; activation through self-ligands or viral RNA fragments may maintain this inflammatory process, suggesting a key role in cancer progression[66]. Chronic activation of TLRs expressed by tumor cells from CRC and pluripotent PROM1 $(CD133)^+$ colon cancer initiating cells may sustain inflammation responses, mediate resistance to apoptosis, and promote further tumor progression. Therefore, targeting of TLR signaling may be a potential mechanism to abrogate this inflammation-mediated effect in tumor progression[66]. The pathways that are downstream of TLRs and culminate in proliferation and recruitment of inflammatory cells during injury can be usurped to support cancer development[70].

Although much effort has been put forward to determine TLR ligand requirements and receptor activity, many questions remain. However, there are reasons to be optimistic that TLRs represent strong candidates for cancer targeting. Drug candidates are being developed to target CRC or act as vaccine adjuvants. We hope that they can be safely used systemically and have the power to transform chemotherapeutic interventions in CRC in the near future.

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TOPIC HIGHLIGHT

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Achieving the best bowel preparation for colonoscopy

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Abstract

Bowel preparation is a core issue in colonoscopy, as it is closely related to the quality of the procedure. Patients often find that bowel preparation is the most unpleasant part of the examination. It is widely accepted that the quality of cleansing must be excellent to facilitate detecting neoplastic lesions. In spite of its importance and potential implications, until recently, bowel preparation has not been the subject of much study. The most commonly used agents are high-volume polyethylene glycol (PEG) electrolyte solution and sodium phosphate. There has been some confusion, even in published meta-analyses, regarding which of the two agents provides better cleansing. It is clear now that both PEG and sodium phosphate are effective when administered with proper timing. Consequently, the timing of administration is recognized as one of the central factors to the quality of cleansing. The bowel preparation agent should be administered, at least in part, a few hours in advance of the colonoscopy. Several low volume agents are available, and either new or modified schedules with PEG that usually improve tolerance. Certain adjuvants can also be used to reduce the volume of PEG, or to improve the efficacy of other agents. Other factors apart from the choice of agent can improve the quality of bowel cleansing. For instance, the effect of diet before colonoscopy has not been completely clarified, but an exclusively liquid diet is probably not required, and a low-fiber diet may be preferable because it improves patient satisfaction and the quality of the procedure. Some patients, such as diabetics and persons with heart or kidney disease, require modified procedures and certain precautions. Bowel preparation for pediatric patients is also reviewed here. In such cases, PEG remains the most commonly used agent. As detecting neoplasia is not the main objective with these patients, less intensive preparation may suffice. Special considerations must be made for patients with inflammatory bowel disease, including safety and diagnostic issues, so that the most adequate agent is chosen. Identifying neoplasia is one of the main objectives of colonoscopy with these patients, and the target lesions are often almost invisible with white light endoscopy. Therefore excellent quality preparation is required to find these lesions and to apply advanced methods such as chromoendoscopy. Bowel preparation for patients with lower gastrointestinal bleeding represents a challenge, and the strategies available are also reviewed here.

Key words: Colonoscopy; Optimal preparation; Polyethylene glycol; Sodium phosphate; Special patients; Timing

Core tip: Bowel preparation for colonoscopy is a central issue related to the quality of the procedure. There are different agents for bowel preparation that can be administered with different schedules. We review the most commonly used agents, as well as new agents and combinations. Moreover, certain considerations should be taken into account for special populations in order to improve safety, efficacy and tolerance. Regimens for bowel preparation in special situations are discussed, such as for pediatric patients, patients with diabetes or inflammatory bowel disease, and in cases of heart or kidney failure or lower gastrointestinal bleeding.

Parra-Blanco A, Ruiz A, Alvarez-Lobos M, Amorós A, Gana JC, Ibáñez P, Ono A, Fujii T. Achieving the best bowel preparation for colonoscopy. *World J Gastroenterol* 2014; 20(47): 17709-17726 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17709.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17709

INTRODUCTION

Colonoscopy is the method of choice to evaluate colonic mucosa and the distal ileum, and plays an important role in diagnosis and treatment. Its successful implementation depends on many factors, but colon cleansing is a key factor[1]. Proper cleaning is usually defined as one that allows the detection of colonic polyps 5 mm or larger[2], though this concept does not consider the shape of the lesions, and it is well known that flat lesions are harder to detect. The cecal intubation rate and adenoma detection rate are two of the main quality endoscopic indices, both of which are directly related to the quality of preparation[3]. Insufficient cleaning can result in lower detection rates of incipient and advanced adenomas, flat lesions, and flat adenomas[3-6], a higher rate of canceled procedures with increased costs, lengthier procedures, and a higher risk of complications[7]. Bowel preparation is one of the issues that negatively influence the willingness of patients to undergo colonoscopy screening[8,9]. Adherence to preparation is a key factor for improving bowel preparation. However, it has limitations due to side effects and poor tolerance among patients to the taste, which are the main reasons for avoiding the procedure[10]. At present, there is no consensus on the ideal method of bowel preparation. This review will analyze different methods of bowel preparation currently available, factors associated with the quality of cleansing, and preparation in special settings, including urgent colonoscopy for lower gastrointestinal bleeding.

WHAT IS THE BEST AGENT FOR BOWEL PREPARATION?

Information from meta-analyses regarding polyethylene glycol and sodium phosphate

The two most widely studied formulations are solutions based on polyethylene glycol (PEG) and sodium phosphate (NaP). PEG electrolyte solution (PEG-ELS) was introduced by Davis *et al*[11] in 1980 and consists of an isotonic oral, non-digestible and non-absorbable solution. Typically, 4 L of PEG-ELS is administered; the high volume and the unpleasant taste are among the major disadvantages of this solution. In the late 1980s, NaP was introduced as an alternative low-volume solution[12]. NaP is a saline laxative administered in two doses of 45 mL diluted in 250 mL water each. Due to its mechanism of action, safety precautions should be taken with patients with a history of or risk of developing renal dysfunction[13].

Many clinical studies have attempted to determine which of these preparations provides better results in terms of colon cleansing, adherence and safety. However, the results are controversial and multiple methodological problems limit the value of comparison. In order to synthesize and critically analyze this information, several meta-analyses of randomized clinical trials (RCTs) have been published, which we will review below.

Six meta-analyses were published between 1998 and 2012 that compared PEG and NaP for bowel preparation of adult patients for elective colonoscopy[14-19] (Table 1). Three of these were developed exclusively to evaluate this relationship[14,17,18], whereas the other three included comparisons of different schedules of the same agent, or the use of other agents. Meta-analyses have also been published comparing different formulations of PEG[20] that are not considered here. The meta-analyses included between 8 and 104 RCTs. Five of the six meta-analyses found no significant difference in quality between PEG and NaP[18]. The outcomes of these meta-analyses considered effectiveness in terms of colon cleansing, tolerance, compliance and security. The main results and characteristics of these studies are summarized.

Effectiveness

Three of the meta-analyses[14,15,18] concluded that NaP is better than PEG in achieving satisfactory colon cleansing (excellent or good: defined as the presence of small volumes of clear liquid in the lumen: < 25%, allowing for viewing more than 90% of the surface)[17]. One of the meta-analyses evaluated PEG and NaP in various presentations and concluded that NaP (in tablet form) was superior to other modalities[18]. The other two studies found no statistically significant differences among preparations. However, in their latest meta-analysis, which included the

Table 1 Features of meta-analyses that compared polyethylene glycol to sodium phosphate

Ref.	Period of inclusion	Comparisons	Trials, *n*[1]	Patients, *n*[1]	Results
Hsu and Imperiale[14]	1980-1996	PEG *vs* NaP	8/8	1286/1286	NaP better than PEG: Better at cleansing Better at compliance Lower cost Safety: NaP = PEG
Tan *et al*[15]	1990-2005	PEG/NaP/sodium picosulfate	29/18	6459/3484	NaP better than PEG: Better at cleansing Better at compliance Safety: NaP = PEG
Belsey *et al*[16]	Until January 2006	PEG/NaP/Others	82/25	-/3748	Cleansing: PEG = NaP NaP better for tolerance Safety: PEG = NaP
Juluri *et al*[17]	1990-2008	PEG *vs* NaP	18/18	2792/2792	NaP better than PEG: Better at cleansing Better at compliance Safety: NaP = PEG
Juluri *et al*[18]	1990-2008	PEG *vs* NaP	71/71	10201/10201	Not statistically different NaP more likely to comply better
Belsey *et al*[19]	Until June 2010	PEG/NaP/others	104/31	-/4450	PEG = NaP PEG better than NaP in proximal colon No information about compliance

[1]total/PEG *vs* NaP. PEG: Polyethylene glycol; NaP: Sodium phosphate.

largest number of studies, Belsey *et al*[19] showed that PEG achieved better cleansing of the ascending colon [odds ratio (OR) = 2.36; 95% confidence interval (CI): 1.16-4.77; $P = 0.012$], which is very relevant for colon cancer screening. They also found that PEG is better when the preparations are fully administered the day before the procedure (OR = 1.78; 95%CI: 1.13-2.8; $P = 0.006$). Unfortunately, all the meta-analyses concluded that there is a wide heterogeneity among the studies included in their reviews, in relation to the small number of trials, poor information regarding randomization methods, route of administering the solution, time between completing preparation and beginning colonoscopy, indication and adherence to fiber-free diet before the procedure, and the lack of validated scales to define colonic cleansing. This last aspect is very important, because this assessment will be influenced by the subjectivity of endoscopists, leading to wide inter-observer variability that limits the validity of the results[7,21].

Compliance

Five meta-analyses evaluating this topic concluded that patients who received NaP have higher rates of success than those receiving PEG[14-18]. As noted above, PEG normally requires high volumes (4 L). It also has a disagreeable flavor that provokes intolerance. Both factors result in lower success rates. However, available information suggests that there is no statistically significant difference when PEG administered in split doses or smaller volumes is compared to NaP[18].

Safety

In general, the studies in the meta-analyses excluded patients with comorbidities such as renal failure, recent myocardial infarction, cirrhosis with ascites, congestive heart failure, acute inflammatory bowel disease, bowel obstruction, etc. With these exclusions, four meta-analyses suggest that there is no statistically significant difference in the profile of clinically significant adverse effects[14-17]. PEG is associated with higher rates of nausea, vomiting and bloating, while NaP has higher incidence of dizziness and mild biochemical abnormalities (hypernatremia, hypocalcemia, hyperphosphatemia, hypokalemia), without clinically relevant impacts[16]. Phosphate-containing solutions have the drawback of side effects and may cause electrolyte problems (hyperphosphatemia, hypocalcemia, hypokalemia, plasma hyperosmolarity, hyponatremia and hypernatremia)[22]. Therefore, their use is discouraged in patients with impaired renal function, dehydration, hypercalcemia or hypertension requiring drug inhibitors of angiotensin converting enzyme, as these patients have experienced phosphate nephropathy related to age, and the dose of the drug[23-25]. Recent guidelines do not support the use of NaP[1].

In summary, overall results from available studies do not indicate that either agent is better than the other, while sub-analyses show PEG to be somewhat better. Although NaP seems to be more tolerable than high-dose PEG, concerns about safety significantly limit the applicability of this agent.

ARE THERE ANY ADVANTAGES TO LOW-VOLUME SOLUTIONS?

Although high-volume PEG formulations are more effective and safer than other osmotic agents, the main disadvantage is the large volume (4 L) that patients are required to ingest and the salty taste due to sodium sulfate. To improve its tolerability, flavored PEG solutions have been

developed (with no sulphates), while low-volume PEG (2 L) has also come into use[17,26].

In a randomized study, a low-volume PEG (2 L) preparation combined with bisacodyl was similar to a standard full-volume PEG preparation in terms of efficacy, but was better tolerated[27]. Five RCTs (a total of 1997 patients) used a commercially available formulation of 2 L of PEG with ascorbate (PEG-A) instead of the conventional 4-L dose of PEG[28-32]. No significant differences were found between the low-volume and the 4-L formulations in terms of cleanliness for the entire colon. However, cleanliness in the right colon (assessed in a single study) was less often satisfactory with the 2-L than with the 4-L PEG (54% *vs* 82% of patients, $P < 0.001$)[30]. Of note, cleanliness in the right colon can be particularly important in screening. Two RCTs reported that willingness to repeat bowel preparation was higher with the low-volume formulation than with the 4-L PEG (73% *vs* 65%, $P = 0.079$)[29,30]. In another randomized study comparing PEG-A to 4-L PEG plus simethicone, no differences were observed in efficacy, safety or tolerance[33]. In a RCT that compared PEG-A to another new low-volume solution (PEG-citrate-simethicone), both in combination with bisacodyl[34], the latter preparation was more effective in bowel cleansing for outpatient colonoscopy. The two low volume solutions were similar in terms of levels of tolerability, safety, acceptability and compliance. However, in this study, the agents were administered on the day before the colonoscopy, which does not comply with current recommendations[1].

PEG-A has also been compared to NaP solutions. In a randomized study, adequate cleansing was obtained in 63.9% with NaP solution *vs* 72.5% with PEG-A[35]. Tolerance was higher with PEG-A. PEG-A has a high level of ascorbic acid (approximately 250 times the recommended daily allowance), which potentially causes hydroelectrolitic-metabolic disturbances. Nevertheless, a recent study showed that PEG-A is similar to 4 L of PEG in terms of safety and hydroelectrolitic changes, except for blood bicarbonate levels, which were lower with PEG-A, though within safe limits[36].

Both magnesium citrate and sodium picosulfate (MC-SP) are also low-volume solutions that should be administered with sufficient liquid to prevent side effects. A combination of SP magnesium oxide and citric acid is commercially available. It can effectively clean the bowel in 70%-80% of patients, but may be associated with dehydration and electrolyte problems[26]. As magnesium is removed exclusively by the kidneys, caution should be exercised in patients with renal failure. Several randomized trials comparing MC-SP preparations with an aqueous NaP preparation found that the SP-based preparations were better tolerated and produced a similar degree of cleansing[37-39]. One trial in which the preparation agents were administered the day before colonoscopy found that right colon cleansing was better with MC-SP plus bisacodyl than with NaP and MC-SP alone[40]. This study suggests that bisacodyl should be added if osmotic solutions are to be given the day before.

MC-SP and PEG preparations have also been compared. One trial found that day-before dosing of MC-SP and a PEG preparation were similar regarding bowel cleansing, but the former was better tolerated by patients[41]. A recent meta-analysis that includes most of the RCTs described showed that 4-L split-dose PEG is better than other bowel preparation methods for colonoscopy[42]. In a related study that compared split dosing of MC-SP with day-before dosing of a PEG plus bisacodyl preparation, patients receiving the SP-based preparation had better colon cleansing and reported better tolerance of the preparation[41,43].

Administering enemas, bisacodyl, or metoclopramide in addition to the standard dose of PEG has not been shown to improve the quality of the preparation or the patient's tolerance, so it is not recommended[44,45]. However, bisacodyl does improve the effectiveness of the preparations of low-volume PEG (2 L)[46].

An alternative to NaP is sodium sulfate. There are still very few studies evaluating this new preparation. A study comparing 4-L PEG solution (given on the day before) to sodium sulfate (given in two doses, the second on the day of the colonoscopy)[22] observed better preparation with the latter (adequate: 71.4% *vs* 34.3%; $P < 0.001$) with no difference in adverse effects. In a pilot study of a Japanese population, sodium sulfate was effective in cleansing the colon in 98% of the cases[47]. Comparison studies including low-volume solutions are shown in Table 2.

A KEY FACTOR FOR OPTIMAL PREPARATION: TIMING OF ADMINISTRATION

The timing of bowel preparation is among the major factors related to the quality of cleansing. However, this has been recognized only recently. In Japan, bowel preparation has consistently been administered on the same day as the colonoscopy[48], usually only a few hours in advance, and often in the endoscopy unit. In contrast, Western countries have not introduced this concept until recently. In 1997, Frommer[49] was the first author to argue that NaP achieved a better quality of cleansing when administered on the same day, and in 1998, Church[50] argued the same regarding PEG. The argument is that gastric and intestinal secretions from the small to the large bowel continue, so that with time, the benefits of bowel cleansing are undone.

In a meta-analysis of randomized trials comparing a full dose of PEG (on the day before colonoscopy) with a split dose (second dose on the same day), Kilgore *et al*[20] found that a split dose significantly increased the rate of satisfactory preparation, the willingness to repeat the same preparation, and significantly decreased the number of discontinued preparations and incidences of nausea. It is not unexpected that when bowel prep is administered the day before colonoscopy, the quality of cleansing

Table 2 Comparison studies including low-volume solutions

Ref.	Comparison	*n*	Conclusion
DiPalma *et al*[27]	PEG 4 L *vs* PEG 2 L+ 20 mg bisacodyl	93/93	PEG 2 L + bisacodyl is more tolerable
Jansen *et al*[28]	PEG 4 L	91/91/102/86/91	PEG 2 L + ascorbate equal to PEG 4 L solution in cleansing quality, taste and compliance
	vs PEG 4 L + with 20 mL simethicone *vs* PEG 2 L + ascorbate *vs* PEG 2 L + ascorbate with 20 mL simethicone *vs* NaP		NaP inferior to PEG 4 L in bowel cleansing quality
Pontone *et al*[29]	PEG 4 L *vs* PEG 2 L + ascorbate	72/72	Residual stool score significantly lower with PEG 4 L
Corporaal *et al*[30]	PEG 4 L *vs* PEG 2 L + ascorbate	149/158	PEG + ascorbate less effective in right colon cleansing
Marmo *et al*[31]	PEG 4 L *vs* PEG 2 L + ascorbate	435/433	PEG + ascorbate as effective as high-volume PEG-electrolyte solution but has superior palatability
Ell *et al*[32]	PEG 4 L *vs* PEG 2 L + ascorbate	153/155	PEG + ascorbate same efficacy and safety, better tolerance
Gentile *et al*[33]	PEG 4 L *vs* PEG 2 L + ascorbate	60/60	Similar efficacy
Repici *et al*[34]	PEG 2 L + ascorbate *vs* PEG 2 L + citrate + bisacodyl	202/203	PEG 2 L + citrate + bisacodyl more effective for bowel cleansing
Bitoun *et al*[35]	PEG 2 L + ascorbate *vs* NaP	169/171	PEG + ascorbate at least as efficacious as NaP, comparable efficacy, better tolerability profile
Rex *et al*[22]	4 L PEG SF-ELS *vs* NaP	68/68	NaP superior bowel cleansing, similar tolerability
Renaut *et al*[37]	MC-SP *vs* NaP	32/41	MC-SP better tolerated, similar cleansing effectiveness
Choi *et al*[38]	NaP *vs* magnesium citrate + NaP (45 mL)	79/80	Both similar effectiveness
Schmidt *et al*[39]	MC-SP *vs* NaP	182/190	MC-SP better tolerance, similar cleansing effectiveness
Hookey *et al*[40]	MC-SP + bisacodyl *vs* MC-SP *vs* NaP	105/109/101	MC-SP + bisacodyl better colon cleansing in the right colon compared with two other groups
Tjandra *et al*[42]	MC-SP *vs* NaP	120/102	NaP better cleansing
Katz *et al*[41]	MC-SP *vs* PEG 2 L + 10 mg bisacodyl tablets	300/303	Similar quality of cleansing
Rex *et al*[43]	PEG 2 L + bisacodyl 5 mg *vs* picosulphate	304/297	Picosulphate is better for cleansing bowel and tolerated

MC-SP: Magnesium citrate and sodium picosulfate; NaP: Sodium phosphate; PEG: Polyethylene glycol; SF-ELS: Sulfate-free electrolyte; SPS: Sodium picosulphate.

is poorer on the right side of the colon[5]. There has been some confusion regarding the relationship between the timing of colonoscopy and the quality of bowel cleansing. Some reports have found that afternoon colonoscopies had superior quality cleansing, but this happened when bowel prep was given on the previous day for morning colonoscopies, and on the same day for afternoon colonoscopies; therefore, in the former, the preparation-colonoscopy interval was longer. For instance, Sanaka *et al*[51] found inadequate bowel preparations in 15% of morning colonoscopies compared to 20% in afternoon colonoscopies when the patients received the preparation the day before. In a study that randomized patients for afternoon colonoscopies to receive 3.8 L of PEG administered the day before, or on the morning of the colonoscopy, the Ottawa score per segments and overall was significantly better with the latter group[52]. Moreover, when the time interval from the moment of administration of the bowel prep to the colonoscopy remains stable, the quality of bowel prep is similar for morning or afternoon examinations. Eun *et al*[53] compared 4-L PEG administered at 5 am for morning colonoscopies, or at 8 pm for afternoon examinations and found similar results in terms of quality of cleansing, noting that colonoscopies performed within 7 h of initiation of PEG intake and those performed within 4 h of completing PEG intake had better quality bowel cleansing. This time interval is accepted as adequate for same-day preparation. When the patients have been prepared the day before, the time interval is different. One study used an intensive preparation strategy (4-L PEG plus a regular dose of NaP), with a median time interval from the last dose of the preparation agent to the start of colonoscopy of 13.5 h[54]. In this study, only 14% of examinations had excellent quality cleansing and 38% good quality. Beyond 14 h after the last dose of the agent, there were no patients with good or excellent cleansing. Therefore, as Eun *et al*[53] pointed out in their paper, the timing of bowel preparation, rather than that of the colonoscopy, determines the quality of cleansing.

One study gave an "intensive" bowel preparation schedule (low fiber diet for 3 d, liquid diet the day before, 10 mg bisacodyl, 3 L of split PEG) to patients with poor cleansing previously (most of the cases with preparation given the day before)[55]. With this split regimen, adequate preparation was achieved in over 90% of the patients. Although there was no control group, this study suggests the importance of preparation timing on the quality of cleansing.

There may be some misunderstanding regarding the right timing for bowel preparation. It is often heard that split-doses should be the rule. However, more importantly than split-doses, we should keep in mind the concept of "same day" preparation, regardless of whether the agent is administered partly or wholly on the same day, and consider that at least half of the preparation should be administered on the same day, a few hours before the colonoscopy[56]. A study compared the quality of cleansing with patient tolerance of 2 L of PEG-ELS, ad-

ministered either on the same day or in a split dose fashion[56,57]. There was no difference in quality (with adequate bowel prep in > 90% patients in both groups). However, patients prepared on the same day only had significantly lower incidence of abdominal pain, slept better, and experienced less interference with their workday the day before.

Several concerns may dissuade doctors from recommending same-day preparation to their patients; first, the relatively short interval (2-5 h) recommended after ingesting prep agent can result in a risk of aspiration. However, there is empirical evidence against this from the Japanese experience[48]. Moreover, Huffman *et al*[58] compared the gastric content of patients receiving only an upper endoscopy, and those undergoing both an upper and lower endoscopy, who received the bowel preparation either on the day before or on the same day. The mean gastric content was slightly reduced in patients undergoing only an upper examination (14.6 mL), but there was no difference in patients that had drunk the bowel prep, regardless of when it was administered (split or on the previous day), and the mean volume was approximately 20 mL.

Although the guidelines of the American College of Gastroenterology recommend split-bowel or same-day preparation for anyone undergoing screening colonoscopy, this may not be the common practice[1,56]. A survey in 2010 (unpublished data) on bowel preparation practices in Spain found that only 15% of the centers gave the preparation, at least in part, on the same day for morning outpatient colonoscopies, whereas 81% of the centers gave the preparation on the same day for afternoon colonoscopies. Physicians often assume that their patients would not be willing to follow a recommendation of split doses[59]. Nevertheless, in a survey study in the US, when patients were explained the importance of the same-day schedule, over 85% were willing to wake up during the night to drink the second dose of a split preparation, and 78% of those who had early morning appointments actually did so[60].

There is abundant evidence indicating that bowel preparation should be administered at least in part on the same day as the examination, in relation to effectiveness of bowel cleansing and detection of neoplasms. Therefore, strategies to improve tolerance and adherence to this schedule should be sought, but patients (and physicians) should receive information about the importance of complying with the instructions for such preparation. However, in spite of the strong evidence available, it is still possible that a patient rejects drinking the preparation on the same day. There is no study specially designed to help provide an adequate preparation for patients receiving the agent on the day before the colonoscopy. There are several factors that could facilitate a better preparation quality in that situation. First, the study by Siddiqui *et al*[54] showed that when the interval beteween the preparation and the start of colonoscopy exceeds 13 h, the quality of cleansing becomes worse. Therefore, the interval should be reduced as much as possible, and should never be longer than 13 h. Secondly, as will be explained later in this article, a low-fiber preparation on the day before the colonoscopy is more patient friendly. Moreover, when patients received a full dose of PEG on the day before the colonoscopy, the quality of cleansing in those who had a fiber free diet the day before was significantly better than in those patients who had a liquid diet[61]. Therefore, a well-designed low-fiber diet should be recommended. Third, in patients who received preparation with MC-SP on the day before the colonoscopy, adding 10 mg bisacodyl two days before the colonoscopy significantly improved the quality of cleansing in the right colon[40]. This adjuvant should probably be employed when bowel preparation cannot be given on the same day as the colonoscopy. Fourth, adding 4 mg loperamide after gut lavage (after liquid stools ceased) in patients who had received the preparation agent on the day before, achieved a significantly better cleansing in the cecum in most of the cases (mean interval from the preparation to colonoscopy around 13 h) in a randomized study. This idea is original and provocative and should be considered as an option, though the results should be confirmed in future studies[62].

TO EAT, OR NOT TO EAT: FACTS ABOUT DIET AND QUALITY OF CLEANSING

Although the type of diet prior to colonoscopy may affect the quality of cleansing, there are surprisingly few studies on this question. Practices vary from no specific pre-exam diet in some Japanese units to liquids-only the day before, which is a common practice in the Unites States. An observational study with inpatients found that the only dietary modification that improved the quality of preparation was a liquids-only diet[63]. Two randomized studies compared the usefulness of a well-defined low-fiber diet to that of a liquid diet the day before colonoscopy. In a Korean study, patients were randomized to a clear liquid diet or a commercial pre-packaged low-fiber diet that includes meals for breakfast, lunch and dinner[64]. Patients received 4 L of PEG in the morning a few hours before colonoscopy. The PEG completion rate was similar in both groups, but satisfaction with bowel preparation was significantly higher with the low-fiber diet. There was no difference regarding adverse events. Moreover, quality of cleansing (Ottawa score) was better in the transverse colon in this group. Another study compared 4 L of PEG the day before with either a liquid or fiber-free diet (which was clearly specified for the different meals) the day before[61]. Interestingly, in this study, the quality of cleansing was better in the fiber-free-diet group, probably because the patients could drink a significantly greater volume of PEG. Moreover, nausea, headaches and vomiting were significantly more common among the liquid-diet group. Another study randomized 230 outpatients receiving a preparation with a low-volume sulfate solution in a split dose, to follow a liquid diet

or a low-residue diet of specified foods the day before colonoscopy[65]. Interestingly, the diet for each meal could be chosen from three options: easy-to-prepare, healthy, or restaurant. There was no difference in the quality of cleansing, but bowel preparation, satisfaction with diet and overall satisfaction were significantly higher with the low-fiber diet. Moreover, the rate of procedure cancellation was higher with the clear-liquid-diet group.

In a descriptive study including patients with poor quality cleansing, an intensive schedule was applied for the repeat colonoscopy, including a liquid diet the day before colonoscopy (and most importantly, with a split dose). The schedule worked well with 90% of patients having adequate cleansing[55]. Therefore, while a well-defined low-fiber diet is generally adequate for outpatient colonoscopy, in certain situations with a high risk of inadequate cleansing, a liquid diet would be more appropriate. More studies in this field are needed.

WILL A MORE INFORMED PATIENT HAVE A BETTER-PREPARED COLON?

Instructions to patients for bowel preparation vary from one endoscopy unit to another and may feature only text (with more or less complex description of the preparation schedule) or include images or figures, with content that may include the importance of colonoscopy in colon cancer prevention, the importance of adequate colon cleansing, dietary recommendations for bowel preparation, and instructions on preparing and drinking cleansing agents. The type and amount of information and the way in which it is delivered to the patient can influence the quality of cleansing. A study in the United Kingdom found that comprehension of a written colonoscopy preparation leaflet was generally low among over 700 patients from different sociodemographic backgrounds, and that health literacy was an independent predictor of comprehension[66]. Ness *et al*[67] found that not following the preparation instructions was, as could be expected, an independent predictive factor for inadequate preparation (OR = 2.61; 95%CI: 1.52-4.75). There was some association with this factor and others associated with inadequate preparation, such as a history of stroke or dementia. Spiegel *et al*[68] meticulously prepared an educational booklet (including images), designed after identifying barriers to colonoscopy preparation. Their patients were randomized to receive standard information or the booklet, and received bowel preparation the day before colonoscopy. Patients that received the booklet had a higher percentage of adequate bowel preparation (68% *vs* 46%; $P = 0.054$). In another study, patients that received bowel preparation in a split-dose system were randomized to receive regular written instructions or additional visual aids[69]. The latter group had better bowel preparation. The importance of providing adequate information to the patients about colonoscopy, bowel preparation, and the importance of following the recommended schedule cannot be overstated.

Bowel preparation is less effective in hospitalized patients. In one randomized study, inpatients received standardized written instructions before colonoscopy, and one group additionally received a 5-min counseling session, explaining the importance of an adequate preparation. This intervention achieved significantly better bowel cleanliness scores[70].

ARE THERE ALTERNATIVE METHODS TO DRINKING AN AGENT TO ACHIEVE GOOD PREPARATION?

The only accepted method for bowel preparation is antegrade ingestion of the agent, and in cases of poor tolerance or impossibility to willingly drink the agent (unconsciousness, dementia, encephalopathy), this antegrade administration can be provided by means of a nasogastric tube. Exclusive preparation with enemas is not accepted as a method of preparation for total colonoscopy, both for efficacy and safety reasons related to risk of explosion if electrocautery is applied[71]. Poor palatability leading to nausea or vomiting can impact patient tolerance and eventually the quality of cleansing[16].

Retrograde bowel cleansing is a promising method for patients with low tolerance or other problems with antegrade cleaning. Until recently, there had few descriptions, however, studies are starting to appear on this issue, probably because of recognition of the importance of colonoscopy for colorectal cancer screening and of bowel preparation for quality colonoscopy. In 1991, Chang *et al*[72] reported a small randomized trial comparing oral PEG (4 L) administered on the previous day to retrograde per-rectal pulsed irrigation with warm tap water by means of a pump. The latter group of patients also received magnesium citrate to facilitate the cleansing of the right colon. There was no significant difference in cleansing quality or other variables of the colonoscopic procedure (time to cecum, aspirated volume, polyp detection).

In 2006, a new device, which consisted of a catheter connected to a pump and water jet, was tested in an animal study. It effectively and safely cleared unprepared animal colons in an average of 12 min[73]. In 2010, another new device was assessed in a relatively large study (57 colonoscopies) with a porcine model[74]. The device consisted of a pump connected to a valve for suction and a disposable part including a tube and a head to the endoscope. The device achieved adequate cleansing in a mean of 4 min. Two studies published in 2012 used the same new catheter-based device to clean the colons of patients with insufficient cleansing at colonoscopy[75,76]. In a comparative study using sequential allocation, Eliakim *et al*[75] applied either the new method or conventional washing with a 50 mL syringe when at least one colonic segment was poorly prepared. The overall colon and cecum-ascending improvement in cleanliness was significantly greater with the new device, while the procedural time was similar. With a device similar in design, Rigaux

et al[76] found better overall and cecum-ascending colon cleansing. Neither study reported on the timing for oral bowel preparation (in the first study, preparation was given the day before, personal communication), but the fact that there was a benefit on the right side of the colon could mean that this segment was especially hard to clean (therefore suggesting that the patients had been prepared the day before). The same year Kiesslich *et al*[77] reported the use of another new device based on a CO_2 pump and a catheter with which CO_2 and saline droplets are applied. In an application with 32 patients, the degree of cleanliness was significantly better, and its use was considered safe.

Horiuchi *et al*[78] applied a 500-mL PEG enema in the hepatic flexure through the working channel of the colonoscope in patients with poor preparation at colonoscopy. Patients were then allowed to go to the lavatory, and needed a mean of 52 min to complete bowel evacuation. Colonoscopy was then repeated, and adequate bowel cleansing was confirmed for 96% of the patients.

Finally, a retrospective study reported the results of another new device for retrograde cleansing. In this study, an evacuation device is inserted in the anus and secured and a sleeve is progressed deep into the colon[79]. Warm water passed by gravity with the water container 2 m above the patient, and by a manual pump. Among the 125 patients who participated in this study, excellent or good quality cleansing was achieved in 89%. Only one patient required sedation during the cleansing procedure because of anxiety, and there were no complications. Fujii[80] recently described a different approach to improve tolerance to bowel preparation by using an antegrade method without the need for patients to drink an agent. Patients who had to undergo upper and lower endoscopy in the same session were included. The proposed method consisted of infusing 1000 mL of PEG-ELS in the second portion of the duodenum (after having completed the diagnostic procedure) with a 50 mL syringe, and then an additional 200-500 mL in the stomach. After completing the upper endoscopy, patients could go to the toilet to complete bowel evacuation, and when the bowel effluent was clean, as confirmed by a nurse, patients underwent colonoscopy. Among the 152 patients who received this preparation method, the quality of bowel cleansing was adequate for 97%, with a mean total time for upper endoscopy of 14 min. There were no complications, and global patient satisfaction with the preparation was excellent for 85% of patients, and moderately satisfactory for 9%. If these results are confirmed in other study populations, this method could be considered for patients with poor tolerance/compliance to standard oral preparations.

HOW TO ACHIEVE THE BEST BOWEL PREPARATION IN SPECIAL SITUATIONS

Inflammatory bowel disease

Complete and good quality mucosal visualization by colonoscopy with intubation of the ileum along with segmental mucosal biopsies is the most valuable tool to distinguish different types of inflammatory bowel disease (IBD), to differentiate IBD from other intestinal disorders, and to determining prognosis and the appropriateness of therapies, along with diagnosis and treatment of complications[81-87].

There have not been adequate studies to determine the best ways to prepare IBD patients for colonoscopy and to identify safety issues associated with different approaches. The lack of research in bowel preparation under inflammatory conditions is therefore surprising, especially for patients who need bowel preparation for repeated examinations[88]. Some publications have found that IBD patients reported low satisfaction from the bowel preparation compared to other patients[89]. Moreover, some ulcerative colitis patients have reported flare symptoms after colonoscopy[90]. The reasons for these negative experiences are unknown, but bowel preparation could be a contributing factor. Clinicians should recognize these side effects of colonoscopy in patients with IBD. The indications of how to prepare these patients prior to colonoscopical procedures are based mostly on expert opinions.

Alternatives for bowel preparation in IBD: Options for bowel preparation include oral PEG-based lavage and oral or enema phosphate. Given that oral NaP solution is associated with frequent aphthoid-like mucosal lesions with missing interpretations, oral PEG is the preferred solution for bowel cleansing[91,92].

The suggested volume of oral PEG is variable, ranging from 2 L to 4 L or more, 6-24 h before the procedure, until reaching the evacuation of a clear fluid[92]. There are no clear recommendations regarding the volume of PEG in the presence of high-volume diarrhea or a high number of bowel movements. However, it seems reasonable to reduce the volume of oral PEG, use a phosphate enema or a combination of both with these patients.

Active IBD setting: In patients with suspected IBD and mildly or moderately active disease, a full colonoscopy along with segmental mucosal biopsies must be performed with formal bowel preparation, preferably using oral PEG. Good bowel cleansing is important in most cases for direct inspection of mucosal patterns of the colon and distal terminal ileum, along with an accurate delineation of the affected location[82,88,93,94].

Severely active IBD: Although colonoscopy appears to be more cost effective than index sigmoidoscopy[95,96], a full colonoscopy with prior bowel preparation is not recommended for patients with acute severe colitis because of the procedural delays and the higher risk of perforation[84-86]. Although a phosphate-enema preparation before flexible sigmoidoscopy is considered safe, it is best to avoid this with patients with dilated colons[83]. A routine administration of an oral purgative can cause colonic dilatation and perforation in severely active disease. A flexible

sigmoidoscopy without bowel preparation or with only a phosphate enema before the procedure can be performed to asses endoscopic criteria of colitis and to obtain biopsies for histologic and cytomegalovirus studies[83,97-99]. In these circumstances, ileocolonoscopy can be postponed until the clinical condition improves[84].

Colonic cancer surveillance: In a recent study, more than a quarter of IBD patients underwent colonoscopies with longer intervals between them than is recommended (> 3-year intervals on average)[100]. One factor that could affect adherence to surveillance colonoscopy is bowel preparation. Detection of a flat lesion against an inflamed background is much more difficult, in part because the quality of bowel cleansing is lower with colitis, with an odds ratio of 0.63 (95%CI: 0.40-0.98)[101]. Good quality preparation with IBD is likely to improve detection rates using mainly oral PEG, especially in cases of remission where the preparation is critical in order to have a reasonable chance of detecting dysplastic lesions[92].

Several novel techniques have been applied to reduce the required number of biopsy samples and the duration of examinations in the context of cancer surveillance, including chromoendoscopy with or without magnification, narrow-band imaging, fluorescence endoscopy, confocal laser endomicroscopy and optical coherence tomography. These novel procedures require perfect bowel cleansing[102].

Small bowel studies: In the context of Crohn's disease, the small bowel must be evaluated. Although wireless-capsule endoscopy and anterograde double-balloon enteroscopy can be performed without bowel preparation, most experts recommend bowel cleansing to certify the presence of small-bowel mucosal changes. It is recommended to use 1.5-2.0 L of oral PEG[103-105]. In a retrograde double-balloon enteroscopy, a standarized bowel cleansing of 2-4 L of PEG is always required[104,106].

Therapeutic IBD procedures: Excellent bowel preparation with a high volume of oral PEG is necessary in therapeutic settings. The main indications are dilatation of benign fibrotic strictures or polypoid resections[107,108]. Gastrointestinal hemorrhage is another possible complication of IBD and the presence of endoscopically treatable lesions, though possible, is uncommon[102].

Elderly patients

Controversies have emerged about the indications for colonoscopy in the elderly. It is known that elderly patients have a higher risk of colorectal cancer[109]. The most common indications for colonoscopy are gastrointestinal bleeding, anemia, changes in bowel habits and abdominal pain. Elderly patients are more likely to have abnormal colonoscopic findings than younger patients[110-112]. In fact, colorectal cancer, vascular and diverticular diseases are more common among the elderly[111,112].

Even though the prevalence of neoplastic lesions increases with age, the diagnostic yield of a screening colonoscopy among the elderly (aged ⩾ 80 years), who have a short life expectancy, is low[113]. This indicates the limits of screening procedures.

Bowel preparation for colonoscopy among the elderly is an important issue when considering the potential benefits and risks of the procedure. A systematic review and meta-analysis observed that in the included studies, poor bowel preparation was documented in 18.8% ± 6.4% of procedures with patients 65 years of age or older, while in patients 80 years or older, poor bowel preparation was reported in 12.1% ± 7.6%[114]. A study of octogenarians showed that tolerance to 4-L PEG was poor in almost 40%[115]. Furthermore, tolerance of bowel preparation was evaluated among elderly patients using either PEG or oral NaP in a retrospective study where patients were subdivided into two groups, one under and the other over 65 years of age, with a mean age of the total group of 60.6 ± 14.8 years[116]. In a separate analysis of adverse events, no significant differences were found between the two preparations, except for nausea, which was experienced by 19% of the PEG group *vs* 39% of the NaP group ($P < 0.009$).

Elderly patients have a higher risk of phosphate intoxication due to a lower glomerular filtration rate, use of medication, and systemic and gastrointestinal diseases. NaP induces electrolyte disturbances such as hyperphosphatemia, hypocalcemia and hypokalemia[117]. The frequency and severity of hypokalemia is due to intestinal potassium loss associated with inadequate renal potassium conservation and is apparently more prevalent in frail patients. In a retrospective study with elderly hospitalized patients with significant comorbidities, there was a 9.6% ($P = 0.008$) incidence of significant hypokalemia with PEG-based bowel preparation[118]. However, other studies have suggested that the efficacy of NaP is similar with non-elderly adults and comparable to that of PEG[119,120].

When assessing the safety of bowel preparation, patients in the PEG group showed fewer changes in the indicators of dehydration and in laboratory tests[12,121]. Due to its large volume, PEG is contraindicated for patients with impaired swallowing function, such patients with stroke, dementia and Parkinson's disease, all of which are more common among the elderly. As noted above, recent European guidelines for bowel preparation advise against the routine use of oral NaP for bowel preparation due to safety concerns (strong recommendation, low quality evidence)[1].

As low-volume bowel preparations with PEG have been shown to provide equivalent cleansing with improved tolerability compared to standard PEG bowel preparation for colonoscopy[34], and the use of a split-dose PEG for bowel preparation before colonoscopy significantly improves the number of satisfactory bowel preparations, increased patient compliance, and decreased nausea compared to full-dose PEG[20], low-volume PEG in a split-dose modality appears to be the ideal bowel preparation for the elderly. Thus, according to recent consensus guidelines for

bowel preparation prior to colonoscopy, patients with high risk of electrolyte disturbances (elderly and debilitated patients, patients at risk of hypokalemia or hyponatremia) should undergo pre-assessment[122].

Diabetic patients

Certain conditions of patients, such as diabetes mellitus, are considered a predictor of inadequate bowel preparation for colonoscopy[67,123]. Many studies have reported poor bowel preparation in diabetic patients compared to non-diabetic patients when using either the same or a higher-volume PEG preparation[124] or NaP for bowel cleansing. In the latter study, a significant difference in optimal bowel cleansing was achieved in 70% of diabetics compared to 94% of non-diabetics ($P = 0.002$). There was a significant correlation among diabetic patients between the quality of bowel cleansing and mean age, duration of diabetes mellitus, level of hemoglobin A1c, fasting blood glucose level, and late diabetic complications[125]. Patients with diabetes often have reduced renal perfusion despite normal serum creatinine. It may be necessary to monitor electrolytes after colonoscopy, particularly with patients with cardiac or renal failure.

Patients with renal failure

As mentioned above, fluid and electrolyte shifts can occur as a result of the hyperosmotic nature of NaP preparations[126]. Consequently, NaP purgatives should not be administered to patients with predisposing factors (*e.g.*, electrolyte abnormalities, renal failure, ascites, congestive heart failure, or a history of myocardial infarction) that can lead to adverse events because of NaP-induced hypovolemia and shifts in serum electrolyte levels. Although electrolyte shifts in patients taking oral or tablet NaP preparations are typically mild, transient and asymptomatic, rare cases of clinically significant hyperphosphatemia have been reported, usually in patients with renal insufficiency[127].

Moreover, failure to maintain adequate hydration before, during, and after bowel preparation can increase the risk of severe and potentially fatal intravascular volume depletion-related complications. Inadequate hydration appears to be an important element in the reported cases of fatal dysnatraemia associated with PEG preparations[128] and renal failure associated with NaP preparations[129]. Therefore, adequate hydration should be maintained throughout the entire bowel preparation process, particularly with high-risk patients such as those taking certain concomitant medications, patients with renal failure, and the elderly.

According to the European Society of Gastrointestinal Endoscopy guidelines for bowel preparation for colonoscopy, PEG is the only recommended bowel preparation for patients with renal failure. The delay between the last dose of bowel preparation and colonoscopy should be minimized and no longer than 4 h[1].

Patients with heart failure

PEG preparations have been shown to increase plasma volume of patients with diseases that predispose them to fluid retention[130]. It has been postulated that this adverse effect occurs less often with lower volume preparations, such as the 2-L PEG regimen combined with bisacodyl or the 2-L PEG 3350 solution. Another concern with PEG solutions is hyperkalemia. Although no clinical reports have shown this finding, the small amount of potassium in this solution is worrisome for patients with heart failure who are taking potassium-sparing diuretics or angiotensin-converting enzyme inhibitors[46].

Nevertheless, when one considers the risks of fluid shifts with NaP preparations, which are in any event contraindicated for patients with congestive heart failure, the safest preparation for patients with congestive heart failure is either a low-volume PEG preparation or a split dose of a standard volume of PEG preparation with careful monitoring during and after use. Clinicians should emphasize the importance of continuing cardiac medications during bowel preparation when appropriate.

In summary, because PEG formulations are osmotically balanced and do not induce substantial shifts in fluid and electrolyte levels, they can be safely administered to patients with electrolyte imbalance, advance liver disease, poorly compensated congestive heart failure, or renal failure. However, reports of increases in plasma volume among patients with concomitant diseases known to cause fluid retention suggest PEG preparations should be used with caution with such patients

Pediatric patients

Colonoscopy is a key tool in the diagnosis and management of a variety of gastrointestinal tract conditions affecting children and adolescents. To perform such a procedure, the colon must be as clean as possible to effectively detect bowel pathology. Inadequate bowel preparation can lead to poor colonic visualization, missed lesions, increased procedure time, and possibly the need to repeat the procedure. With pediatric populations, it is one of the most difficult parts of the procedure from the patient's perspective.

Over the years, there have been many bowel preparations for children. There is a wide variability in the type, dose and length of bowel preparations at different institutions. Medications that have been used include lavage solutions (PEG with and without electrolytes), osmotic solutions (magnesium citrate), and laxative cleaning agents (senna, bisacodyl, NaP, and phosphate enemas).

There are only a few comparative studies of different bowel preparations with children. Single published randomized trials with pediatric populations demonstrated high efficiency of both PEG with electrolyte solutions and oral NaP[131-133]. However, oral administration of NaP to children has limitations because of serious adverse effects, such as electrolyte and fluid disturbances and acute kidney injury[133]. On the other hand, PEG with electrolytes solution also presents of the problems of the high volume required and its unpalatability[134-139]. Given theses problems, alternatives have been studied.

Laxative agents such as bisacodyl and senna have

been evaluated with children, used in combination with clear liquid diets for 2-3 d and enemas[140,141]. One RCT showed good bowel cleaning with sennosides, whereas bisacodyl with an enema-based protocol had a high rate of poor preparation (37%), resulting in the need for repeated examinations[141]. Other alternative bowel preparation regimens are based on osmotic agents alone or combined with laxatives[21,131,132,141-148]. Although excellent or good bowel cleansing rates were reported in 40%-100% of the children, depending on the regimen, these studies are mostly non-randomized, with a limited number of patients. As well, they were evaluated based on a subjective assessment of the overall quality of the bowel preparation.

Currently, PEG without electrolytes is the mainstay for treating constipated children. It has been shown to be effective, safe, palatable, and with excellent compliance[149]. Because of these properties, PEG has been studied as a bowel preparation option. Two studies have demonstrated that PEG can be used as a safe and effective preparation for children with a dose of 1.5 g/kg for 4 d[134,150]. However, bowel preparation should ideally be done in a shorter period of time. To establish an effective dose of PEG, a prospective study determined that 1.9 g/kg per day for 2 d with a clear-liquid diet resulted in clear stools in > 90% of patients with excellent/good Aronchick scores[151]. Another prospective study evaluated a 2-d PEG preparation with 2 g/kg per day PEG with bisacodyl supplementation[152]. Although demonstrating efficacy (92% excellent/good cleanliness), the study was not blind, lacked a comparison group, and did not assess safety by measuring electrolytes. Recently, Abbas *et al*[153] reported a prospective open-label study evaluating a 1-d PEG preparation for children. In the study, 46 children were given 238 g of PEG mixed with 1.9 L of Gatorade over a few hours before the colonoscopy. Only 37 children (82%) ingested the full preparation. Nevertheless, all of the colonoscopies were completed to the cecum, and 77% had effective bowel preparation according to the scale used in the study. Adverse clinical effects were common and included nausea/vomiting (60%) and abdominal pain (44%). There were no clinically significant electrolyte changes. The major advantage of this preparation is a short duration, especially useful for emergency colonoscopies.

Terry *et al*[154] recently evaluated the efficacy of PEG and senna for bowel preparation of children. The study was a well-designed blind randomized prospective trial. Thirty patients were randomly assigned to receive PEG at a dose of 1.5 g/kg per day or senna (15-30 mL/d) for 2 d before the colonoscopy. Good/excellent scores for colon cleanliness were given to 88% of patients in the PEG group compared to 29% in the senna group. Both regimens were generally well tolerated without any significant adverse clinical effects or electrolyte changes.

A recent study by Kierkus *et al*[155] included 10-18-year-old patients randomly assigned to receive either PEG 60 or PEG 30 mL/kg per day plus oral bisacodyl 10-15 mg/d (BPEG) or sennosides 2 mg/kg per day for 2 d. Of 240 patients enrolled in the study, 234 patients were available for analysis of the efficacy of colon cleansing. No significant differences were found among the three groups in terms of the proportions of participants with excellent/good (PEG: 35/79; BPEG: 26/79; sennosides 25/76) and poor/inadequate (PEG: 20/79; BPEG: 28/79; sennosides 28/76) bowel preparation evaluated with the Aronchick scale and for the total mean Ottawa score (PEG: 5.47 ± 3.63; BPEG: 6.22 ± 3.3; sennosides: 6.18 ± 3.53). These results showed that high-volume PEG, low-volume PEG plus a laxative stimulant, and sennosides have similar effectiveness and are equally tolerated by patients being prepared for colonoscopy. There were no serious adverse events reported during the bowel cleansing.

Ideal bowel preparations should be effective, safe, and easily accepted by children. It seems that PEG meets these requirements. However, the appropriate duration and dose need to be determined through further randomized and controlled trials.

Patients with acute lower gastrointestinal bleeding

A significant proportion of patients admitted to hospitals have acute lower gastrointestinal bleeding (LGIB). The incidence in the US is about 36/100000 persons, especially among elderly patients that may be taking medications such as anticoagulants or aspirin that interfere with platelet function. Most acute LGIB stops spontaneously without the need for intervention. Furthermore, most cases end without an identified source of bleeding. In such situations there is risk of rebleeding. In more severe episodes of LGIB, it is crucial to identify the source of bleeding; therefore, a therapeutic procedure should be performed. Various studies have identified the most important source of bleeding as diverticula, followed by vascular lesions, both of which can be effectively treated by colonoscopy with good bowel preparation. Some studies have shown that the probability of finding lesions increases with shorter intervals between LGIB and the colonoscopy, though the improvement is not consistent or significant. Consequently, the value of urgent colonoscopy remains controversial[156-159].

Although there have been reports concerning colonoscopy for acute lower bleeding in which no oral preparation was given, it is now widely accepted that oral preparation plus early colonoscopy achieves better diagnostic and therapeutic performance[157,160,161]. Moreover, there is risk of explosion when electrocautery is used in patients with unprepared colons, as about 50% of patients have potentially explosive concentrations of hydrogen and methane[162,163]. To obtain optimal colonic preparation, it is important to first define if an urgent colonoscopy is necessary (performed within hours of admission), which is recommended in more severe cases of LGIB. Different studies have shown that early colonoscopy can reduce the length of hospitalization, which is an important consideration, especially in public hospitals with high demand for beds[159].

Bowel preparation for urgent colonoscopy: One prospective study involved 121 patients with diverticular hemorrhaging that underwent urgent colonoscopy (within 6-12 h). All patients received a PEG purge, two-thirds orally and one-third by nasogastric tube, and all required 5-6 L of purge and 3-4 h to clean the colon. Notably, 7% (two in the urgent group and three in the routine group) required repeat colonoscopies secondary to inadequate preparation[164].

In a study by Green *et al*[165] in 2005, 50 patients that underwent colonoscopy received PEG (a total of 4-6 L, 250 mL every 15 min) orally or by nasogastric tube for patients that could not drink the solution; 3-4 h were necessary to clean the colon. The elective colonoscopy group was prepared with routine 4-6 L of PEG, administered orally beginning the night before the procedure, which was performed within four days of admission. This study did not mention the quality of preparation, rates of cecal intubation or the duration of the colonoscopy, but it was more successful at finding the source of bleeding in the urgent colonoscopy group (42%) than standard colonoscopy (22%) (OR = 2.6; 95%CI: 1.1-6.2). Nevertheless, there was no difference in terms of the need for surgery or the incidence of rebleeding.

In another randomized trial of urgent *vs* elective colonoscopy among patients that had been hospitalized with LGIB, both groups were prepared with PEG (4 L in 3 h and underwent colonoscopy within 12 h in the urgent group). No benefits were found for the urgent colonoscopy group, and once again, no data was mentioned regarding quality of preparation[166].

More recently, a feasibility study was conducted on urgent colonoscopy (6-24 h) without traditional preparation. Thirteen patients with severe LGIB were prepared with a combination of three 1-L water enemas 20 min apart. Immediately after the enemas, patients underwent colonoscopy with a hydroflush technique, combining water-jet irrigation and mechanical endoscopic suction, which allows the use of large volumes of water to lavage the colon (500 mL/min). The researchers obtained adequate endoscopic visualization for definitive or presumptive identification of the source of bleeding in all procedures. Cecal intubation was used in 67% of the cases (in the remaining cases a definite or presumptive origin of the bleeding had been detected), the duration of colonoscopy was 38 min, and mean insertion time was 11 min[167].

In one reported case, an antegrade transendoscopic lavage was applied in a patient with severe lower bleeding, by infusing 4 L of PEG with an irrigation pump at 100 mL/min (over 40 min). This preparation allowed for performing a colonoscopy 8 h later that detected diverticular bleeding[168]. This approach is similar to the method described by Fujii[80] for outpatient colonoscopy with a prospective series of 152 patients.

Thus, to obtain a clean colon in the context of LGIB, it is necessary to use a large volume of PEG (4-5 L on average). Up to 50% of cases may require a nasogastric tube, in which case colon preparation can take 4-6 h. This traditional preparation could be replaced in the future by water-jet techniques that will allow for performing urgent colonoscopy, while avoiding the intake of large amounts of purge or the installation of an NG tube, and likely reducing the length of hospitalization.

CONCLUSION

The importance of an adequate quality of cleansing for colonoscopy cannot be overstated. Efficacy, tolerance, and safety have to be considered when choosing the agent for each patient. The schedule of administration, including timing and the diet chosen, has implications for the quality of cleansing. It is imperative to inform the patient about the importance of colonoscopy and the preparation method, as it is clear now that good information leads to better quality of preparation. Finally, special characteristics of the patients, including comorbidity, must be considered in order to provide them with the safest and more effective method of bowel preparation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (17): Intestinal microbiota

Impact of the gut microbiota on rodent models of human disease

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Abstract

Traditionally bacteria have been considered as either pathogens, commensals or symbionts. The mammal gut harbors 10^{14} organisms dispersed on approximately 1000 different species. Today, diagnostics, in contrast to previous cultivation techniques, allow the identification of close to 100% of bacterial species. This has revealed that a range of animal models within different research areas, such as diabetes, obesity, cancer, allergy, behavior and colitis, are affected by their gut microbiota. Correlation studies may for some diseases show correlation between gut microbiota composition and disease parameters higher than 70%. Some disease phenotypes may be transferred when recolonizing germ free mice. The mechanistic aspects are not clear, but some examples on how gut bacteria stimulate receptors, metabolism, and immune responses are discussed. A more deeper understanding of the impact of microbiota has its origin in the overall composition of the microbiota and in some newly recognized species, such as *Akkermansia muciniphila*, Segmented filamentous bacteria and *Faecalibacterium prausnitzii*, which seem to have an impact on more or less severe disease in specific models. Thus, the impact of the microbiota on animal models is of a magnitude that cannot be ignored in future research. Therefore, either models with specific microbiota must be developed, or the microbiota must be characterized in individual studies and incorporated into data evaluation.

Key words: Animal models; Gut microbiota; Diabetes; Obesity; Cancer; Allergy; Behavior; Colitis

Core tip: Full characterization of the gut microbiota of animal models has revealed that animal models within different research areas, such as diabetes, obesity, cancer, allergy, behavior and colitis, are highly affected by their gut microbiota. The mechanistic aspects are not clear; however, the impact of the microbiota on animal models is of a magnitude that cannot be ignored in future research. Therefore, either models with specific microbiota must be developed, or the microbiota must be characterized in individual studies and incorporated into data evaluation.

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INTRODUCTION

Host-microbiota relationship

The gut is an ideal incubation chamber for bacteria adapted to the mammal body temperature and the anaer-

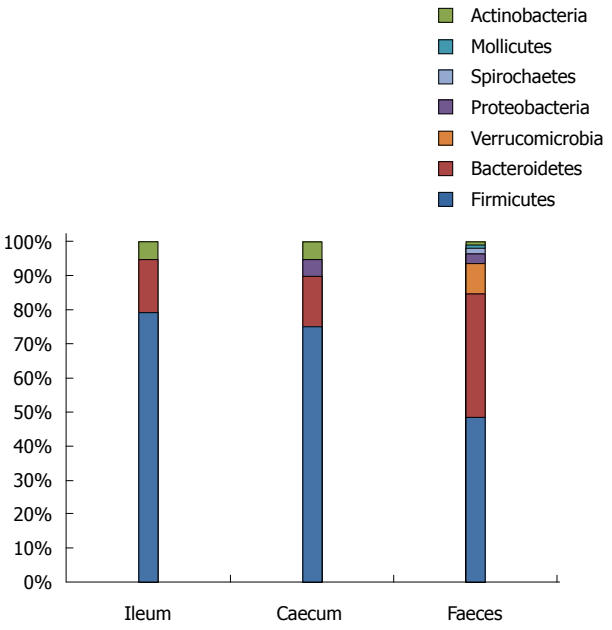

Figure 1 The approximate composition of the gut microbiota in the ileum, cecum and feces of mice[3,7,8,11,16].

obic environment. Thousands of years of co-existence has led to such adaptation, and the mammal gut harbors 10^{14} organisms dispersed over approximately 1000 different species, dependent on how the cut-offs are set for similarity. Within the traditional approach to laboratory animal bacteriology, bacteria have been considered as either pathogens, commensals or symbionts; however, there seems to be a need for a broader understanding of this. When first inside the gut, the bacteria will be fed and will be allowed to propagate, while the host organism will benefit from otherwise unavailable products of microbial digestion. Generally, pathogenicity is not in the interest of the microorganism, because it induces a strong and eradicating immune response from the host, and even in the case of microbial victory in this battle, the end result may be the death of the host and the need for the microbe to relocate to a new habitat. The host immune system, on the other hand, needs to protect the host from invasion without being so aggressive that it loses the microbe and thereby all its benefits.

Complexity of microbial impact on the host

A more advanced understanding of the impact of the microbiota takes into consideration both the overall composition and the balance between the members of the microbiota, as well as some newly recognized species, which, by themselves, seem to have an effect on the specific models. Some of these have a symbiotic effect, while others push disease development in a more detrimental direction. However, same species may act in favor of the development of one disease, while being more protective against another disease, and the mechanistic potential of the species may differ between different parts of the gut. For most of these bacteria, it is their abundance, rather than their qualitative presence or absence, which are responsible for their effect on the host[1-4]. The microbiota is normally not very diverse in the upper part of the gut, *e.g.* in the ileum, where there is a huge accumulation of lymphatic tissue available for stimulation[3,5-10]. It gradually becomes more diverse as the gut contents pass through the large intestine and become feces (Figure 1)[3,5-11]. In both man and mouse, a microbiota with a low diversity is indicative of an increased risk of developing inflammatory disease[12,13]. Furthermore, in animals, a microbiota that is roughly similar in the upper part of the gut, may differ substantially in the lower part of the gut and *vice versa*[3,14]. Finally, there might be essential differences between the effects of the various species at different ages of the animals, which may explain why some species favor the development of one disease, while protecting against another.

Modern microbiological identification techniques

Over recent decades, new methods based upon molecular biology diagnostics have been developed. Such methods, which include quantitative real-time polymerase chain reaction (qPCR) assays[15], pyrosequencing[16] and metagenomic sequencing, have permitted identification of close to 100% of the gut's operational taxonomic units (OTU), which include both cultivable and non-cultivable bacterial species, and in principle, viral, eukaryotes and Archea[17], although they are seldom specifically tested for at present. In contrast, previous cultivation techniques only allowed cultivation and identification of 10%-20% of the bacterial species present in the gut[18]. These molecular biology-based tools have enabled detailed correlation studies. Such studies have revealed that a range of animal models within a range of different research areas are affected by their gut microbiota[19].

GENERAL MECHANISMS UNDERLYING THE GUT MICROBIOTA EFFECT

As described below, the impact of the microbiota on animal models is well documented, while the mechanisms underlying this are less clear. Some hypotheses, though, make more sense than others. As techniques for the full characterization of the microbiota have been developed over the last decade, we are only now beginning to achieve an understanding of how the microbiota actually exerts its effect on the host; however, some examples can be given.

Window of opportunity

In early life, there is a window for the induction of oral tolerance in the gut[20]. This seems essential to avoid inflammatory disease later in life[21]. Molecular structures in bacteria known as microbial-associated molecular patterns (MAMP) stimulate pattern-recognition receptors (PRR) in the host, thereby inducing innate responses[22]. Among the most important PRRs are the toll-like receptors (TLR), which are present in different types on a range of different cell types[22-29] (Figure 2). An impor-

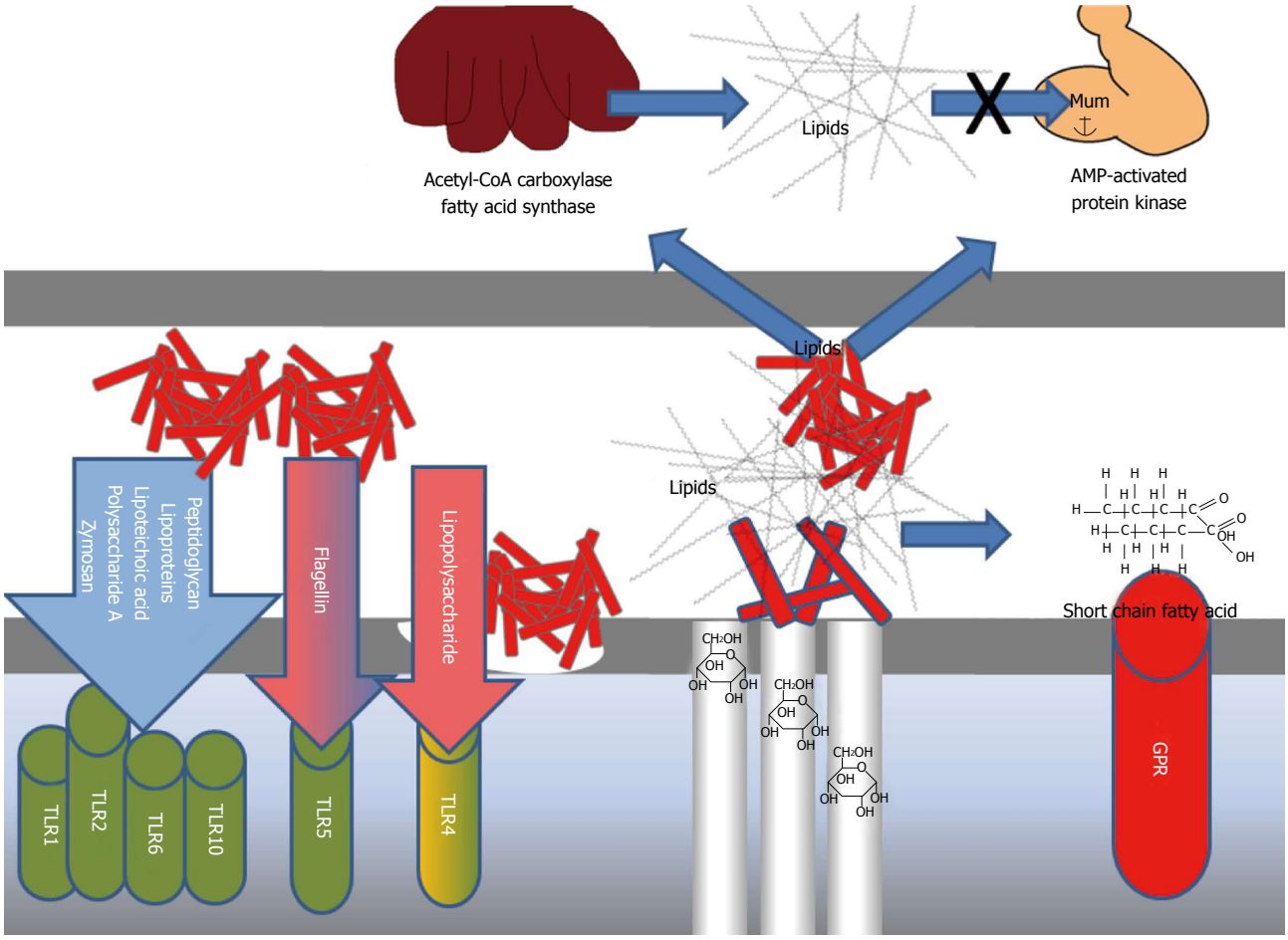

Figure 2 Examples of some theories on potential pathways for the impact of gut microbiota on animal models of human disease. Bacterial colonization may double the density of capillaries in the small intestinal epithelium, thereby promoting intestinal monosaccharide absorption[28]. Undigested food components may be fermented into SCFAs and subsequently act as signals for GPRs of importance for the development of obesity[26,29]. Bacteria may express several key enzymes relevant for hepatic lipogenesis[27,50], and hepatic and muscular fatty acid oxidation[31]. Molecular structures in the cell walls of bacteria may act as MAMPs, which stimulate TLRs on the host cells to induce innate immune responses. The complex of TLR1, TLR2, TLR6 and TLR10 is expressed on a range of cell types such as enterocytes, macrophages, dendritic cells, natural killer cells, mast cells, T cells, B cells, neutrophilic cells and Schwann cells, and may be stimulated by various MAMPs, *e.g.* peptidoglycan, from Gram positive bacteria cell types[21,23-25,31-33]. TLR4, expressed by *e.g.* macrophages, dendritic cells, mast cells, natural killer cells and enterocytes, is stimulated by lipopolysaccharides from Gram negative bacteria[30], while flagellin from various bacteria may stimulate TLR5 expressed by *e.g.* mucosal dendritic cells and macrophages[33]. Mucin-degrading *Akkermansia muciniphila* may reduce the mucus layer to increase TLR-stimulation[79]. SCFAs: Short chain fatty acids; GPR: G-protein receptor; MAMP: Microbial-associated molecular pattern; TLR: Toll-like receptor.

tant example of a MAMP is lipopolysaccharides (LPS), which are important parts of the cell wall of Gram negative bacteria[30], such as Proteobacteria[31], from which it stimulates TLR4. Another important example is peptidoglycan, found in the cell walls of Gram positive bacteria, which stimulates TLR2[32] and flagellin deriving from flagellated bacteria, leading to stimulation of TLR5[33]. Therefore, as different types of MAMPs stimulate different TLR's dispersed on a variety of different cell types[23], and as MAMPs are also dispersed and shared between members of the microbiota[22], there is a vast range of innate host responses to bacteria.

Adult life stimulation

The age of the animal also makes a difference. For example, stimulation of TLR1, TLR2 and TLR4 in early life leads to higher production of interleukin (IL)-6 than stimulation later in life[34]. Germ free animals have more T helper cells type 2 (TH2) and less TH1 cells[35], as the stimulation of the gut lamina propria dendritic cells, *e.g.* by polysaccharide A (PSA) from *Bacteroides fragilis,* induces IL-12 secretion, which favors TH1 at the cost of TH2[36]. Host-bacterial interactions, probably mediated through glucagon-like peptide 2 (GLP-2), seem to control the gut barrier function[37]. Metabolic endotoxaemia is responsible for the phenomenon whereby excess intake of dietary fat increases plasma LPS levels[38,39], which in mice, is a sufficient molecular mechanism to trigger metabolic diseases, such as obesity and diabetes[40].

EXAMPLES OF SOME ANIMAL MODELS UNDER IMPACT OF THE GUT MICROBIOTA

Impact of germ free status

The clearest documentation of a general microbial impact on rodent models is observed when comparing a conven-

Table 1 Examples of rodent models in which germ free status has a documented impact

Model	Disease
Models with increased disease incidence or severity	
β-lactoglobulin induced mouse[51]	Allergy
NOD mouse[42]	Type 1 diabetes
MyD88 KO NOD mouse[42]	Type 1 diabetes
Restrained mouse[43]	Stress
Models with decreased disease incidence or severity	
Ovalbumin-specific TCR TG mouse[44]	Allergy
Swiss-Webster mouse[45]	Anxiety
Collagen induced rat[52]	Arthritis
HLA-B27 TG rat[53]	IBD
IL-2 KO mouse[54,55]	IBD
IL-10 KO mouse[56]	IBD
TCRα KO mouse[57]	IBD
Dextran sulfate sodium induced mouse[46]	IBD
SAMP1/Yit mouse[47]	IBD
Adoptive T-cell transfer in the mouse[48]	IBD
Carrageenan, LPS, or formalin induced mouse[49]	Inflammatory pain
C57BL/6 mouse[65]	Obesity
C57BL/6 mouse[65]	Type 2 diabetes

NOD: Non-obese diabetic; MyD88: Myeloid differentiation primary response gene 88; KO: Knockout; TCR: T cell receptor; TG: Transgenic; HLA-B27: Human leucocyte antigen subtype B27; IL-2: Interleukin 2; SAMP1/Yit: Senescence accelerated mice prone line 1 Yakult; LPS: Lipopolysaccharide. IBD: Inflammatory bowel disease.

tional model with a microbiota with a germ free version. In several studies, this has revealed essential differences in disease expression (Table 1)[22,41-57]. Although germ free mice eat more, they are leaner, and they have less body fat compared with conventional mice because they are less efficient in extracting energy from their diet[50]. Germ free mice have increased expression of obesity-related peptides, such as glucagon-like peptide 1 (GLP-1) in the brain[58], which is relevant, because central GLP-1 reduces food intake in rats[59]. Germ free mice also behave differently from microbiota-harboring mice and this behavior may be normalized by colonization[43]. However, for this phenotype there also seems to be an important time window in early life[60]. Germ free mice with a mutation causing a defect in the skin barrier suffer from a more severe B-lymphoproliferative disorder, because they express significantly higher levels of the proinflammatory cytokine thymic stromal lymphopoietin[61]. Inflammatory bowel disease (IBD) occurs either because of a TH1/TH17 response (Crohn's disease) or a TH2 response (ulcerative colitis) to gut commensals[62]. Therefore, IBD under germ free conditions does not develop at all in, *e.g.* Human Leucocyte Antigen subtypes B27 (HLA-B27) transgenic rats[53] and IL-10 knockout mice[56]. For the IL-10 knockout mice[63] it does not occur even under barrier protected conditions (Table 1). IL-2 knockout mice may, under germ free conditions, show mild focal intestinal inflammation[64] (Table 1).

Impact of fluctuations in the gut microbiota composition

Within animal models of the metabolic syndrome, there seems to be an association between the gut microbiota and at least some of the metabolic parameters. For example, in leptin-deficient obese mice, there is a strong correlation between glycated hemoglobin levels and the composition of the gut microbiota[1]. Further, these mice have significantly more Firmicutes and fewer Bacteriodetes members compared with their wild-type and heterozygous litter mates[10]. Their obese phenotype may be transferred with the microbiota by recolonizing germ free lean wild-type mice[65]. In C57 Black substrain 6 (C57BL/6) mice on both high and low calorie diet, continuous oral ampicillin improves glucose tolerance[66,67]. However, this effect is mainly caused by an early life impact on glucose tolerance, and the effect ceases immediately after termination of treatment; thereafter, the glucose tolerance may even decrease[68,69]. Several studies describe cross-talk between the brain and the gut through both the vagal system and the hypothalamus-pituitary-adrenal (HPA) axis[70]. Stressing animal models changes their microbiota[71], and the composition of the gut microbiota has an impact on responses in rodent stress tests[72,73]. Innate immune system cytokines, such as IL-1, IL-6 and tumor necrosis factor α (TNFα), which may originate from a gut microbiota provocation, induce "sickness behavior", changing the priorities of the organism to enhance recovery and survival[74]. However, metabolites formed by microbial decomposition in the gut may also have a direct impact on the brain[75]. In mouse models of atopic dermatitis, more than 70% of the variation observed in the local tissue cytokine response may be shared with the variation in gut microbiota[76]. Changes in the structure of the microbial community seem to reduce the number, as well as the size, of tumors in azoxymethane/dextran sodium sulfate (AOM/DSS) colon cancer-induced mice, and tumor induction may be achieved by colonizing germ free mice with microbiota from induced mice[77].

EXAMPLES OF THE IMPACTS OF SPECIFIC BACTERIAL SPECIES

Verrucomicrobioa

Akkermansia muciniphila (*A. muciniphila*) is a Gram negative bacterium, which in mice is the only species belonging to the phylum Verrucomicrobia[78]. It interacts *via* its mucin degrading capabilities with enteroendocrine cells to modulate gut barrier function, and it is capable of producing certain short chain fatty acids (SCFA's) with a direct action on the receptor G-protein receptor 43 (GPR43)[79]. Abundance of *A. muciniphila* is reduced in mice with obesity and type 2 diabetes[80], and it gradually disappears as aging leptin deficient obese mice develop insulin resistance[1]. In non-obese diabetic (NOD) mice it becomes more abundant when mice are fed a gluten-free diet, which decreases the incidence of type 1 diabetes[81]. Early life treatment with vancomycin in NOD mice allows *A. muciniphila* to become a dominant gut microbiota member, which reduces the incidence of type 1 diabetes[3], but enhances susceptibility to allergic asthma[82], which

is in accordance with other studies showing allergy and diabetes to counteract one another in NOD mice[83,84]. Induction of IBD in mice with dextran sodium sulfate (DSS) reduces the number of extracellular vesicles derived from *A. muciniphila*, and feeding DSS induced mice such vesicles reduces the severity of IBD[85], which fits well with observations in humans[4]. However, it not only reduces the severity of diseases: its presence is correlated with higher severity when infecting mice with *Salmonella typhimurium*[86], and AOM/DSS colon cancer-induced mice have an increased abundance of *A. muciniphila*[77], which may be explained by its ability to downregulate the natural killer cell receptor, NKG2D, which is part of the anti-carcinogenic defense[87].

Firmicutes

Segmented filamentous bacteria (SFB's) are clostridia-related Gram-positive bacteria[88]. The term has been applied for decades to describe intestinal bacteria of a uniform morphology[89]. However, today the term refers to one single species, also known as *Candidatus Savagella*[90]. SFBs induce secretion of the pro-inflammatory cytokine IL-17 from TH17 cells[91], which in the adult mouse is correlated with a low number of regulatory T cells[92]. The presence of SFB's differs between mice from different vendors[92], and SFB positive NOD mice have a significantly lower incidence of type 1 diabetes compared with SFB negative ones[93]. In the adoptive transfer severe combined immune deficiency (SCID) mouse model of IBD, SFBs are essential for the induction of severe inflammation[48]. Furthermore, SFBs and the induced TH17 are important in the defense against intestinal pathogens. For example, mice infected with *Citrobacter rodentium*, a potent murine colon pathogen, exhibit severe symptoms if they lack SFBs[91].

IBD in IL-10 knockout mice is enhanced by *Enterococcus fecalis*[94,95], which is probably linked to its production of gelatinase[96].

Faecalibacterium prausnitzii (*F. prausnitzii*) is a clostridia-related bacterium[97] linked to a protective effect against human Crohn's disease[98]. Oral feeding of *F. prausnitzii* reduced the severity of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice, and some studies indicated that this may also be the case in both multidrug resistance gene deficient (*mdr1a* knockout)[99] and in the DSS-induced mouse models of colitis[100].

High abundances of *Lactobacillus* spp. and bifidobacteria are correlated strongly with low levels of inflammation in mice[101] and leptin in rats[102], which also fits well with these bacteria acting protectively against IBD in IL-10 knockout mice[103], allergic sensitization in mice[104], and myocardial infarction in rats[102]. *Lachnospiraceae* seems quantitatively correlated to improved glucose tolerance in leptin-deficient obese mice[1].

In stressed mice, there is correlation between their Firmicutes levels and their responses in the stress tests[73]. Ingestion of *Lactobacillus rhamnosus* in mice regulates their emotional behavior and central γ-aminobutyric acid (GABA) receptor expression *via* the vagus nerve[72].

Bacteroidetes

A high abundance of the Gram negative family Prevotellaceae, perhaps restricted to one unclassified genus, in the gut of leptin-deficient obese mice correlated with impaired glucose tolerance[1].By contrast, in AOM/DSS induced colon cancer mice, a high abundance of Prevotellaceae correlated with a low tumor burden[77]. *P. copri*, which has been correlated with the development of arthritis in humans, seems to increase the severity of DSS induced colitis in mice[5]. *Caspase-3* knockout mice exhibit a lower inflammatory response to DSS induction of colitis compared with wild-type mice; however, this protective effect of the mutation is decreased by cohousing knockout mice with wild-type mice, which significantly increases the abundance of *Prevotella* spp. in the knockout mice[105].

Bacteroides vulgatus seems to enhance IBD in HLA-B27 transgenic rats[106] and IL-10 knockout mice[95], and in the Bio Breeding (BB) rat, a spontaneous type 1 diabetes model. The fecal microbiota differ and contain an increased number of *Bacteroides* spp. before onset of diabetes[107]. As in all other mammals, *Bacteroides* spp. form an important part of the Bacteroidetes fraction of the rodent gut[16]. These Gram negative bacteria are important for the processing of complex molecules to simpler ones in the gut[108]: complex glycans are their key source of energy[109]. *B. fragilis* toxins cause symptoms of diarrhea and IBD in germ-free mice[110], and they induce colonic tumors strongly in multiple intestinal neoplasia (MIN) mice[111]. On the other hand, *B. fragilis* PSA, which is important for the inflammatory gut response to pathogens[36], also protects against *Helicobacter hepaticus*-induced colitis in mice; probably *via* the prevention of IL-17 secretion[112]. Feeding the maternal immune activation (MIA) mouse model with *B. fragilis* reduces symptoms of autism, which is probably linked to the normalization of the levels of a specific gut metabolite[113].

The abundance of *Alistipes* spp., a bacterium of the Rikenellaceae family, seems to increase when mice are stressed by grid floor housing[73].

Proteobacteria

Escherichia coli (*E. coli*) enhances IBD in HLA-B27 overexpressing rats[106], although *E. coli* Nissle stabilizes the enteric barrier in mice[114]. When reducing type 1 diabetes by pre-weaning treatment of NOD mice with vancomycin, a vast increase in the abundance of Proteobacteria in the pups was observed[3].

Actinobacteria

Bifidobacterium spp. in rodents have a positive impact on the regulatory and innate immunity[101,115]. Perinatal supplementation of *B. longum* reduced TH1 and TH2 responses in allergen sensitized mice[104]. On the other hand, their numbers are also increased in gluten-fed NOD mice with a high incidence of type 1 diabetes compared with NOD

mice on a gluten-free diet[81].

DISCUSSION

The information gained over the last decade on how the entire microbiota, as well as some of its individual members, affect animal models of very different types, has prompted the scientific community to incorporate this in future production and quality assurance of animal models. It is not possible to regard these matters from a "Specific pathogen-free" concept, as some of the species act in favor of the development of one disease, while against the development of another disease, *e.g.* SFB's both protect against type 1 diabetes and induces a T_H17 response in favor of the development of Crohn's disease. Furthermore, the balance between the different fractions of the microbiota is also likely to make a difference. Ultimately, it is often a quantitative rather than a qualitative presence that makes the difference. Therefore, it is likely that we will see more tailor-made rodent models, *i.e.* commercial breeders and research groups have sought to produce animals with a specific microbiota for the conditions under test. One obvious idea may be to breed such animals by selective breeding; however, this does not seem to increase the microbiota similarity, although the microbiota of offspring show a clear clustering with the mother's microbiota[116,117]. It is probably rational to inoculate germ free mice with a tailor-made microbiota around weaning, as they are conventionalized in SPF conditions[118]. The window for induction of oral tolerance in animal models may also be turned around, such that a low bacterial stimulation in the open phase of this window may be essential to develop target diseases in the model. When stimulated later on, the nature of this stimulation is also essential, because commonly used disease models in rodents are driven by specific subsets of T cells[19]. Another alternative will be to characterize the microbiota composition for animals in sensitive studies and incorporate this in the data evaluation by chemometric or multifactorial statistical means. The impact of the gut microbiota on animal models is of a magnitude that cannot be neglected in the future.

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TOPIC HIGHLIGHT

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Intestinal microbiota and type 2 diabetes: From mechanism insights to therapeutic perspective

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Abstract

The incidence of type 2 diabetes (T2DM) is rapidly increasing worldwide. However, the pathogenesis of T2DM has not yet been well explained. Recent evidence suggests that the intestinal microbiota composition is associated with obesity and T2DM. In this review, we provide an overview about the mechanisms underlying the role of intestinal microbiota in the pathogenesis of T2DM. There is clear evidence that the intestinal microbiota influences the host through its effect on body weight, bile acid metabolism, proinflammatory activity and insulin resistance, and modulation of gut hormones. Modulating gut microbiota with the use of probiotics, prebiotics, antibiotics, and fecal microbiota transplantation may have benefits for improvement in glucose metabolism and insulin resistance in the host. Further studies are required to increase our understanding of the complex interplay between intestinal microbiota and the host with T2DM. Further studies may be able to boost the development of new effective therapeutic approaches for T2DM.

Key words: Intestinal microbiota; Type 2 diabetes; Body weight; Bile-acid metabolism; Insulin resistance; Intestinal microbiota modulation

Core tip: Type 2 diabetes (T2DM) is believed to be caused by a series of multiple risk factors such as genetic liability, age, overweight or obesity, and an unhealthy lifestyle. Recently, accumulated evidence has suggested that the intestinal microbiota plays an important role in the pathogenesis of T2DM as a potential novel contributor. This review focuses on the underlying role of intestinal microbiota in the pathogenesis of T2DM and the therapeutic potential of modulating gut microbiota in T2DM.

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INTRODUCTION

According to recent estimates by the International Diabetes Federation, there are 382 million people living with diabetes worldwide, and the number is expected to rise to 592 million by 2035[1]. Nearly 85%-95% of people with diabetes have type 2 diabetes (T2DM)[2]. T2DM is believed to be caused by a series of multiple risk factors such as genetic liability, age, overweight or obesity, and an unhealthy lifestyle. Recently, accumulated evidence has suggested that the intestinal microbiota plays an important role in the pathogenesis of T2DM as a potential novel contributor.

The adult human intestine is colonized by about 100 trillion bacteria, which is about 10 times the number of total cells in the human body[3]. Recent evidence suggests that the intestinal microbiota composition is associated with obesity and T2DM. Ley *et al*[4] analyzed 5088 bacte-

rial 16S rRNA gene sequences from the gut microbiota of obese *ob/ob* mice and their lean control group. They found that *ob/ob* mice had a 50% decrease in the abundance of Bacteroidetes and a proportional increase in Firmicutes. They also observed similar differences in the gut microbiota of obese compared with lean humans[5]. Intestinal microbiota compositional changes have also been investigated in patients with T2DM. Researchers have found that the abundance of Firmicutes and Clostridia was significantly reduced, while the relative proportion of Bacteroidetes and Betaproteobacteria was increased in the diabetic group compared with the control group[6]. However, Zhang *et al*[7] found that the proportion of Firmicutes and Clostridia were higher in the group of patients with T2DM compared to the normal glucose group. Patients in the pre-diabetes and T2DM groups had a significantly increased level of Betaproteobacteria compared with the normal glucose group. Qin *et al*[8] have developed a protocol for a metagenome-wide association study based on deep shotgun sequencing of the gut microbial DNA extracted from fecal samples from Chinese T2DM patients and nondiabetic controls. They identified 47 metagenomic linkage groups in the T2DM-associated gene markers from the gut metagenome. Their results showed that patients with T2DM had a moderate degree of gut microbial dysbiosis, a reduction in the abundance of some butyrate-producing bacteria, and an increase in various opportunistic pathogens. Karlsson *et al*[9] observed significantly higher levels of four *Lactobacillus* species and significantly lower levels of five *Clostridium* species in the T2DM group. Importantly, these changes did not correlate with body mass index (BMI), waist circumference, or waist-to-hip ratio. Sato *et al*[10] showed that stool samples of diabetic patients had significantly reduced levels of the *Clostridium coccoides* group, *Atopobium* cluster, and *Prevotella*, and a significantly increased level of total *Lactobacillus* compared with control subjects. They also noted that the detection rate of live gut bacteria in the blood of diabetic patients was significantly higher than that in control subjects (28% *vs* 4%, $P < 0.01$). These studies that have aimed to evaluate the association between gut microbiota and diabetes have produced conflicting results. There may be many factors influencing the results, such as race, eating habits, geographical location, and research methods. This review focuses on the underlying mechanism of intestinal microbiota in the pathogenesis of T2DM, and the therapeutic potential of modulating the gut microbiota in T2DM.

INTESTINAL MICROBIOTA AND BODY WEIGHT

Humans do not have the enzymes necessary for digestion of many types of plant polysaccharide, such as cellulose, xylans, resistant starch, and inulin[11]. However, these indigestible carbohydrates can be fermented by intestinal microbes to yield energy and to produce short-chain fatty acids (SCFAs). The role of the intestinal microbiota in the regulation of host body weight and energy homeostasis was revealed primarily in rodents. Bäckhed *et al*[12] and his colleagues found that conventionally raised mice had 42% more total body fat than germ-free mice (raised in the absence of any microorganisms), even if their daily caloric intake was 29% less than their germ-free counterparts. The germ-free mice transplanted with fecal microbiota from conventionally raised animals for 14 d had a 57% increase in their total body fat. In further investigation, the fecal gross energy content of lean conventionally raised C57BL/6J mice was significantly higher than in their obese counterparts (*ob/ob* mice)[13]. Germ-free mice transplanted with fecal microbiota from obese donors had a significantly greater increase in total body fat than those colonized with microbiota from lean donors. These results confirmed that the intestinal microbiota in *ob/ob* mice was more effective in harvesting energy from food than that of their lean littermates.

INTESTINAL MICROBIOTA AND BILE-ACID METABOLISM

Cholic acid and chenodeoxycholic acid are the primary bile acids synthesized from cholesterol in the liver in humans. Once the primary bile acids, such as cholic acid and chenodeoxycholic acid, have reached the intestine, they may be transformed by intestinal microbiota into secondary bile acid species: deoxycholic and lithocholic acids[14]. The intestinal microorganisms strongly affect bile acid metabolism. The most typical secondary bile acid and the most abundant bile acid in biliary bile in humans is deoxycholic acid, which is converted from cholic acid *via* a 7α-dehydroxylation reaction catalyzed by some species of *Clostridium* in the large intestine[15]. Compared with germ-free mice, conventionally raised (CONV-R) mice have significantly lower levels of bile acid in the gallbladder and small intestine, but significantly higher levels of bile acid in the cecum, colon, feces, and serum. The total amount of bile acid was 71% lower in CONV-R than germ-free mice. Activation of the nuclear farnesoid X receptor (FXR) by the gut microbiota reduce the expression levels of most bile acid synthesis enzymes[16]. In turn, bile acids contribute to suppression of bacterial colonization and growth in the gut because of their strong antimicrobial activity. A previous study showed that the primary mechanism underlying the antimicrobial action of bile acids was membrane damage[17]. Only microbial populations able to tolerate physiological concentrations of bile acids can survive in the gut. Feeding with cholic acid induces phylum-level alterations in the composition of the gut microbiota in rats. Cholic acid feeding increases significantly the ratio of Firmicutes to Bacteroidetes, which is similar to the changes induced by high-fat feeding[18].

Over the past decade, a growing body of evidence has shown that bile acids play an important role in glucose metabolism as signaling molecules and cellular

receptor ligands. Bile acids activate not only FXR but also the membrane-bound, G-protein-coupled receptor (GPCR) 1 (also known as TGR5)[19]. It has been demonstrated that bile acids inhibit the expression of gluconeogenic genes, such as those encoding phosphoenolpyruvate carboxykinase, fructose-1, 6-biphosphatase-1, and glucose-6-phosphatase *in vitro via* FXR[20]. Knocking out FXR in *ob/ob* mice prevented diet-induced obesity and improved murine hyperglycemia and glucose tolerance by increasing peripheral glucose clearance and adipose tissue insulin sensitivity[21]. Bariatric surgery, such as vertical sleeve gastrectomy (VSG), is effective for treatment of obesity and comorbid T2DM. FXR contributes to the maintenance of weight loss and improvement in glucose tolerance following VSG, which are associated with increased circulating bile acids and transition in gut microbiota composition[22]. Activation of TGR5 in enteroendocrine L cells induces glucagon-like peptide (GLP)-1 release, with an improvement in liver and pancreatic function and increased glucose tolerance in obese mice[23]. Activation of TGR5 in brown adipose tissue and muscle increases energy expenditure and alleviates diet-induced obesity[24].

Fasting serum taurine-conjugated bile acid concentrations are higher in T2DM compared with normoglycemic controls, and intermediate in impaired glucose tolerance. This pattern is not directly linked to obesity and glucose *per se*[25]. However, increases in taurine-conjugated bile acid in patients with T2DM may be related to lower rates of taurine deconjugation that is catalyzed by some bile salt hydrolases enriched in the human gut microbiota[26]. Bile-acid sequestrants have been used to sequester bile acids in the intestine to increase bile acid synthesis and consequently reduce serum low-density lipoprotein cholesterol. Bile-acid sequestrants have also been demonstrated to improve glucose control in patients with T2DM, which might be through multiple mechanisms such as altering the bile acid pool composition, improving hepatic glucose metabolism, increasing release of incretin hormones, and inducing changes in gut microbiota composition[27,28].

INTESTINAL MICROBIOTA AND INSULIN RESISTANCE

Endotoxemia and low-grade inflammation

Obesity, insulin resistance and T2DM are closely associated with low-grade inflammation characterized by disordered cytokine production and activation of a network of inflammatory signal pathways[29,30]. The low-grade inflammation is induced by a change in gut microbiota that promotes metabolic endotoxemia and triggers the development of inflammation *via* a lipopolysaccharide (LPS)- and CD14/toll-like receptor (TLR) 4-dependent mechanism[31] (Figure 1). The intestinal microbiota is enriched with molecules such as LPS and peptidoglycan that may cause inflammation. Gut microbiota-derived LPS is involved in the onset and development of inflammation and metabolic diseases[32]. Elevated plasma LPS concentration in mice induced by high-fat feeding is defined as metabolic endotoxemia. Moreover, a high-fat diet in mice significantly alters intestinal microbiota composition. Metabolic endotoxemia is induced in mice through continuous subcutaneous infusion of LPS for 4 wk, and fasted glycemia and insulinemia, weight gain and expression of inflammatory cytokines are increased similarly to those in mice fed a high-fat diet. CD14 knockout mice resist most of the LPS and high-fat diet-induced characteristics of metabolic diseases. CD14 plays a key role in innate immunity[33]. The binding of LPS to the complex of mCD14 and TLR4 at the surface of the innate immune cells activates the cascade reaction of inflammation[34,35]. Many typical proinflammatory stimuli, including LPS, lipids, fatty acids, and chemokines activate c-Jun N-terminal kinase (JNK) and IκB kinase (IKK)-β pathways intracellularly. IKKβ activation stimulates the family of nuclear factor (NF)-κB transcription factors and increases the expression of numerous mediators of inflammation that can cause insulin resistance. JNK activation promotes the phosphorylation of insulin receptor substrate (IRS)-1 at serine sites, which inhibits normal signal transduction through the insulin receptor/IRS-1 axis, which also can result in insulin resistance[36]. Therefore, the metabolic endotoxemia induced by LPS derived from the gut microbiota is associated with inflammation and insulin resistance.

Gut permeability

Increased endotoxemia is correlated with increased gut permeability. A high-fat diet in mice dramatically increases gut permeability and reduces expression of tight junction proteins such as zonula occludens (ZO)-1 and occludin in intestinal epithelial cells. Antibiotic treatment reduces metabolic endotoxemia in high-fat-fed mice, which is associated with decreased gut permeability, reduced inflammatory markers, and improved metabolic features of diabetes and obesity. Furthermore, the deficiency in CD14 in *ob/ob* CD14 knockout mice demonstrates the metabolic and inflammatory effects similar to those of antibiotics[37]. Intestinal permeability in human T2DM detected by the ^{51}Cr-EDTA urinary recovery test was significantly increased compared with matched control subjects[38]. Modulating gut microbiota composition with prebiotics improves gut permeability, reduces metabolic endotoxemia, lowers inflammation, and alleviates glucose intolerance[39,40].

Endocannabinoid system

The endocannabinoid (eCB) system is now believed to be associated with inflammation and diabetes[41,42]. Intestinal microbiota modulate gut eCB expression, which controls gut permeability and plasma LPS levels through the CB1 receptor[43]. Changes in the gut microbiota due to prebiotic feeding reduce gut permeability in obese mice. Blocking the CB1 receptor in obese mice also improves gut barrier function by increased distribution and localization of tight junction proteins (ZO-1 and occludin).

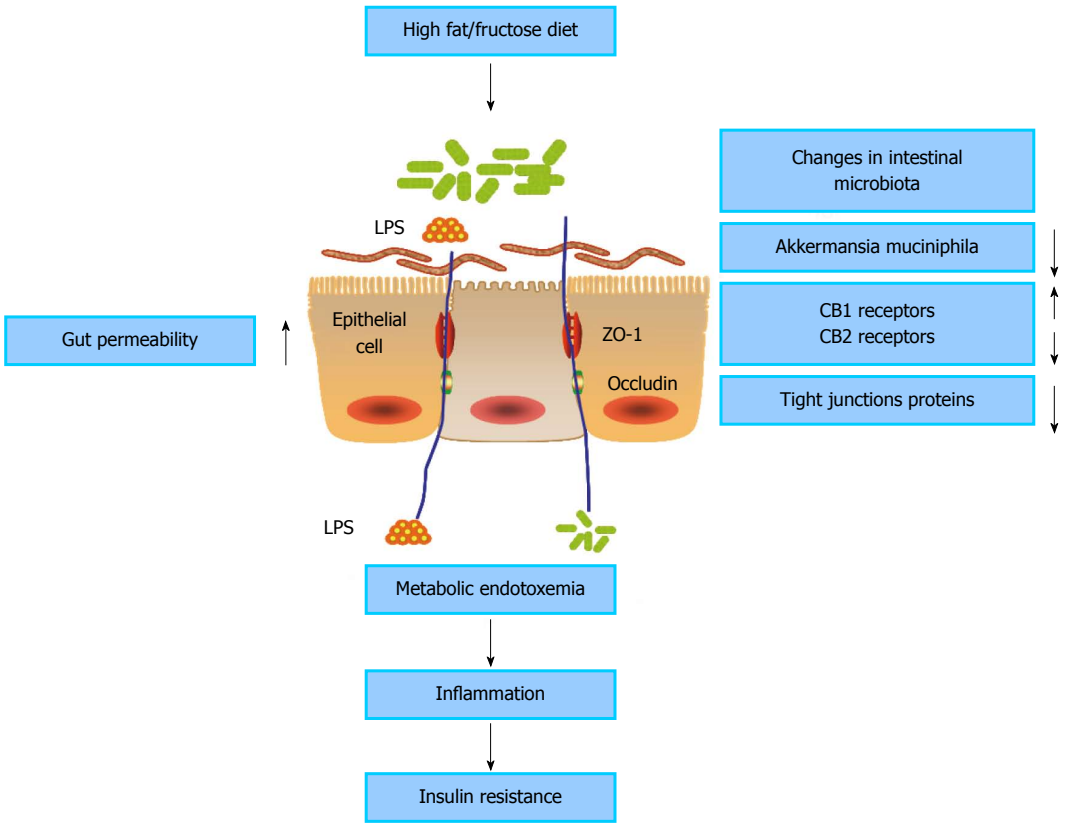

Figure 1 Influence of the intestinal microbiota in promoting gut permeability and insulin resistance. Changes in the intestinal microbiota reduce tight junction proteins of gut epithelial cells and increase gut permeability, thus promoting metabolic endotoxemia and insulin resistance. LPS: Lipopolysaccharide.

This demonstrates that the eCB system modulates gut permeability through the distribution and localization of tight junction proteins[44]. Bermudez-Silva *et al*[45] have shown that cannabinoid CB2 receptor activation improves glucose tolerance in rats and that CB1 receptor blockade mimics the effects of CB2 receptor agonists. The data suggest that the eCB system modulates glucose homeostasis through the interplay of CB1 and CB2 receptors. The changes in CB2 receptor expression are correlated positively with intestinal counts of *Lactobacillus* supplement and negatively with counts of *Clostridium* supplement[46]. Modulation of the intestinal microbiota with specific probiotics has been shown to upregulate CB2 receptor expression in rodents[47].

Akkermansia muciniphila

Akkermansia muciniphila (*A. muciniphila*) is a mucoprotein-degrading bacterium that colonizes the mucous layer[48], and its abundance is negatively correlated with body weight in humans[49,50]. The abundance of *A. muciniphila* decreases in obese and T2DM mice. Feeding with viable *A. muciniphila* ameliorates high-fat-diet-induced metabolic disorders, including adiposity, metabolic endotoxemia, low-grade inflammation, and insulin resistance. Feeding also promotes intestinal expression of eCBs that control inflammation, gut barrier, and gut hormone secretion. However, these effects are not observed in the same mouse model fed with heat-killed *A. muciniphila*[51].

INTESTINAL MICROBIOTA AND NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders, including steatosis, nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis, which is often associated with obesity, insulin resistance, and diabetes mellitus. The prevalence of ultrasonographic NAFLD in patients with T2DM ranged from 54.11% to 78% in different studies[52]. A two-hit mechanism has been proposed for the pathogenesis of NAFLD. The first hit is the process of triglyceride accumulation in hepatocytes, while the second hit is responsible for hepatocyte injury, inflammation, and fibrosis through oxidative stress, lipid peroxidation, and proinflammatory cytokines[53,54]. Accumulated evidence has suggested that intestinal microbiota may be associated with NAFLD progression. Bäckhed *et al*[12] observed that colonization of germ-free mice with normal intestinal microbiota induced insulin resistance and stimulated hepatic lipogenesis. Gut microbiota compositional change can increase the amount of TLR ligands delivered to the liver. TLR ligands stimulate Kupffer cells and hepatic macrophages to produce proinflammatory cytokines that can result in insulin resistance and hepatocyte death[55]. In addition to TLRs, nucleotide-binding oligomerization domain-like receptors (NLRs) are inflammasome-dependent pathways of proinflammatory cytokine production[56,57]. Henao-Mejia *et al*[58]

demonstrated that the NLRP6 and NLRP3 inflammasomes and the effector protein interleukin-18 negatively regulated NAFLD/NASH progression through modulation of the gut microbiota. A meta-analysis to evaluate the effects of probiotic therapy in NAFLD showed that probiotic therapies can improve liver function, lipid metabolism, and insulin resistance in NAFLD patients[59]. A recent randomized clinical trial demonstrated that 4-mo supplementation with probiotics significantly improved fatty liver in children with NAFLD[60].

INTESTINAL MICROBIOTA AND GUT HORMONES

GLP-1

GLP-1 is an incretin secreted from intestinal L cells. GLP-1 has numerous physiological effects, including stimulation of glucose-dependent insulin secretion, augmentation of β-cell mass, and inhibition of glucagon release, gastric emptying, and food intake[61]. Yadav *et al*[62] modulated the gut flora composition of mice with a probiotic, VSL#3. The altered gut microbiota stimulated production of SCFAs (butyrate) that promoted GLP-1 secretion from L cells to improve the metabolic state. SCFA activation of GPCR GPR41 and GPR43 promotes the secretion of GLP-1[63]. Prebiotics are non-digestible dietary ingredients that cause specific gut microbial composition changes or stimulate selectively the activity of some microbial species. Many investigations have demonstrated that prebiotics increase release of GLP-1 and improve the metabolic inflammation and insulin resistance induced by a high-fat diet[64-66].

GLP-2

GLP-2 is co-secreted with GLP-1 and is able to enhance intestinal epithelial proliferation and reduce gut permeability[67]. Changes in mouse gut microbiota with prebiotic ingestion promote a significant release of plasma GLP-2 levels and improve systemic and hepatic inflammation. GLP-2 receptor blockade impairs prebiotic-induced improvement in inflammatory tone[39]. Besides the roles of GLP-2 in maintaining gut barrier integrity, slowing gastric emptying and intestinal motility, improving nutrient absorption, and enhancing immune function, GLP-2 in central neurons enhances hepatic insulin sensitivity and plays a key role in the control of glucose homeostasis[68].

Peptide YY

Peptide YY (PYY) is a gastrointestinal hormone secreted from intestinal L cells. PYY has several biological actions including vasoconstriction, inhibition of gastric acid secretion, reduction of pancreatic and intestinal secretion, regulation of appetite and inhibition of gastric motility[69,70]. Rats receiving a diet supplemented with oligofructose, oligofructose-enriched inulin or high-molecular-weight inulin demonstrated an increase in portal serum levels of GLP-1 and PYY. The same effects were also observed in diabetic rats[71]. Dietary-resistant starch is a fermentable fiber that liberates SCFAs through fermentation in the gut. Feeding rats with dietary-resistant starch increases GLP-1 and PYY secretion in the lower gut through SCFAs[72].

THERAPEUTIC PERSPECTIVE OF INTESTINAL MICROBIOTA MODULATION FOR T2DM

Probiotics and prebiotics

A modulating effect of the gut microbiota on T2DM was suggested by recent observations. Probiotics are non-pathogenic live microorganisms that may confer health benefits on the host. In an animal study, researchers observed that a fermented milk product containing probiotic bacteria significantly delayed the onset of glucose intolerance, hyperglycemia, and hyperinsulinemia in diabetic rats induced by high fructose concentration[73]. In elderly T2DM patients who consumed a daily dose of 200 mL of a symbiotic drink containing 10^8 CFU/mL *Lactobacillus acidophilus*, 10^8 CFU/mL *Bifidobacterium bifidum* and 2 g oligofructose over over 30 d, there was a significant increase in high-density lipoprotein cholesterol and a significant reduction in fasting glycemia[74]. In another investigation, patients with T2DM who consumed 300 g/d of probiotic yogurt containing *L. acidophilus* La5 and *Bifidobacteriumlactis* Bb12 for 6 wk had a significant reduction in fasting glycemia and hemoglobin A1c[75].

Antibiotics

Antibiotic treatment is another method of gut microbiota modulation. Treatment with norfloxacin and ampicillin (1 g/L each) for 2 wk, suppressed the numbers of cecal bacteria in *ob/ob* mice. The treated animals displayed a significant improvement in fasting blood glucose and oral glucose tolerance. The enhanced insulin sensitivity was independent of food intake, weight loss, or adiposity. In this study, both plasma LPS levels and the expression of jejunal tumor necrosis factor-α level were significantly lower in the antibiotic-treated mice than in the control mice, suggesting that modulation of intestinal microbiota by antibiotics ameliorated the inflammatory status in *ob/ob* mice[76]. When diet-induced obese and insulin resistant mice were treated with the non-absorbable antibiotics polymyxin B and neomycin, they had a gradual reduction in glycemia, associated with a modified cecal microbiota profile[77]. Berberine, one of the main ingredients of a Chinese traditional herb used to treat bacterial diarrhea, improves glycemia. The antimicrobial activity of berberine and its modulation of the gut microbiota may play a role in the antidiabetic effect of this herb[78]. However, long-term use of antibiotics in humans is related to weight gain and obesity. Patients who receive 6 wk intravenous treatment with vancomycin plus gentamicin for infective endocarditis show significant weight gain[79]. Hernández *et al*[80] observed that intravenous β-lactam therapy for 14 d promoted glycosidase activity in the hu-

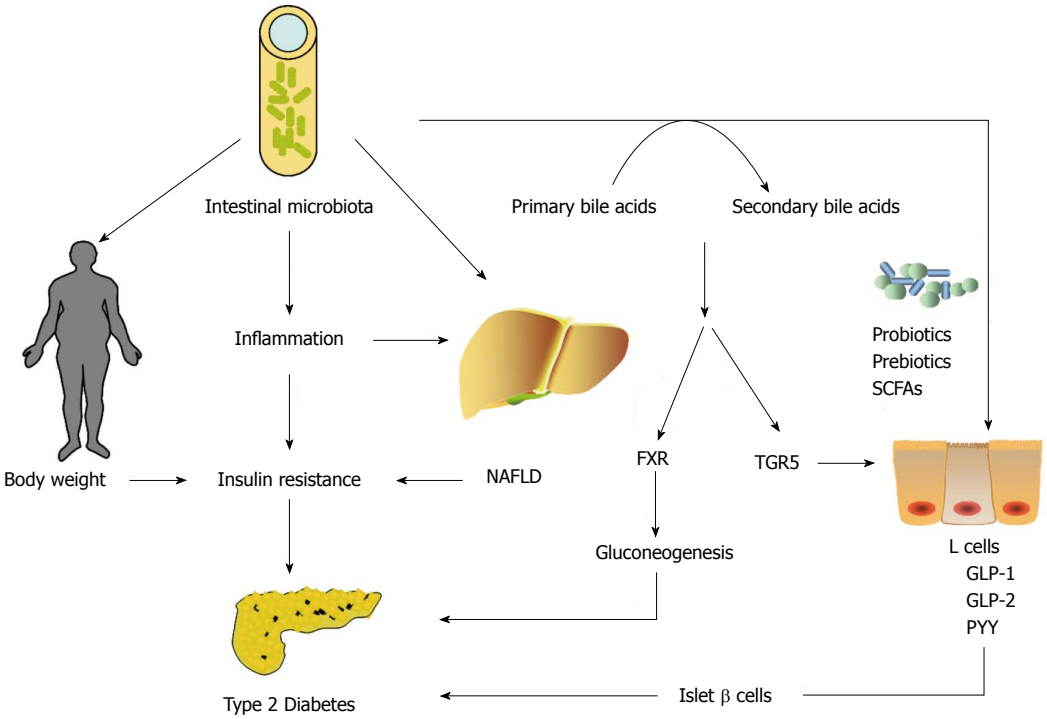

Figure 2 Role of the intestinal microbiota in the pathogenesis of type 2 diabetes. The intestinal microbiota may play an important role in the onset of type 2 diabetes by influencing body weight, bile acid metabolism, proinflammatory activity, NAFLD and insulin resistance, and modulating gut hormones. NAFLD: Non-alcoholic fatty liver disease; SCFAs: Short-chain fatty acids; FXR: Farnesoid X receptor; GLP: Glucagon-like peptide; PYY: Peptide YY.

man gastrointestinal tract and was associated with BMI and glucose level. These data presented an interesting view of the potential effects of antibiotics on human metabolism. Further studies should be performed to investigate the effects of different antibiotics and administration routes on metabolism and T2DM.

Fecal microbiota transplantation

Recently, a report about fecal microbiota transplantation has aroused strong interest. Fecal microbiota transplantation was testified to be a highly successful therapy for recurrent *Clostridium difficile* infection[81] This also raised interest in the therapeutic effect of fecal transplantation in metabolic syndrome and T2DM. Patients with metabolic syndrome who received small intestinal infusions of fecal microbiota from allogenic lean donors for 6 wk showed an improvement in peripheral and hepatic insulin sensitivity, along with an increase in butyrate-producing intestinal microbiota[82].

CONCLUSION

Intestinal microbiota may play an important role in the pathogenesis of T2DM by influencing body weight, bile-acid metabolism, proinflammatory activity and insulin resistance, and modulation of gut hormones (Figure 2). Modulating the gut microbiota through the use of probiotics, prebiotics, antibiotics, and fecal microbiota transplantation may have benefits in improving glucose metabolism and insulin resistance in the host. However, there are still many questions that need to be resolved. LPS inhibits the synthesis of insulin in isolated rat islets of Langerhans through binding of TLR4 and activation of the NF-κB pathway[83]. We do not know whether the changes in intestinal microbiota directly influence the β cell mass and function of islets in T2DM patients. Can we detect gut bacterial genes in liver or islets in T2DM patients? Does bariatric surgery for obese T2DM patients interact with their intestinal microbiota? Rifaximin, an oral locally acting antibiotic used to treat inflammatory bowel disease, can modulate gut microbiota. What effect of rifaximin can we observe if it is administered to T2DM animal models or patients? Future studies are required to increase our understanding of the complex interplay between intestinal microbiota and the host with T2DM, and to enable the development of new effective treatments for T2DM.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (20): Gastrointestinal surgery

Per-oral endoscopic myotomy: Major advance in achalasia treatment and in endoscopic surgery

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Abstract

Per-oral endoscopic myotomy (POEM) represents a natural orifice endoscopic surgery (NOTES) approach to laparoscopy Heller myotomy (LHM). POEM is arguably the most successful clinical application of NOTES. The growth of POEM from a single center in 2008 to approximately 60 centers worldwide in 2014 with several thousand procedures having been performed attests to the success of POEM. Initial efficacy, safety and acid reflux data suggest at least equivalence of POEM to LHM, the previous gold standard for achalasia therapy. Adjunctive techniques used in the West include impedance planimetry for real-time intraprocedural luminal assessment and endoscopic suturing for challenging mucosal defect closures during POEM. The impact of POEM extends beyond the realm of esophageal motility disorders as it is rapidly popularizing endoscopic submucosal dissection in the West and spawning offshoots that use the submucosal tunnel technique for a host of new indications ranging from resection of tumors to pyloromyotomy for gastroparesis.

Key words: Per oral endoscopic myotomy; Achalasia; Natural orifice transluminal endoscopic surgery; Submucosal tunnel endoscopic resection; Submucosal tunnel endoscopy; Endoscopic suturing; Endoscopic myotomy; Endoscopic submucosal dissection; EndoFLIP

Core tip: Per-oral endoscopic myotomy (POEM) is a novel endosurgical therapy for achalasia. POEM developed as an offshoot of early natural orifice endoscopic surgery (NOTES) approach investigation, but, now, is arguably the most successful clinical application of NOTES. The clinical results of POEM therapy in terms of dysphagia relief and safety have been excellent. The impact of POEM is extending far beyond the narrow domain of esophageal motility disorders. As the first successful clinical application of submucosal endoscopy it is now spawning many other NOTES interventions utilizing a submucosal tunnel approach including submucosal tunnel endoscopic resection of submucosal and mucosal lesions and per oral pyloromyotomy for gastroparesis.

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INTRODUCTION

Achalasia is a rarely encountered motor disorder of the esophagus, which is usually of unclear etiology. Most patients have dysphagia to solids and liquids and others may have chest pain, regurgitation, and coughing.

Barium esophageal contrast study usually shows a dilated esophagus tapering to a "bird's beak" with delayed contrast passage into the stomach, but diagnosis is confirmed by esophageal manometry showing infrequent or no relaxation of the lower esophageal sphincter (LES) and a variety of abnormalities in the esophageal body. Medical therapy is ineffective in achalasia and treatment is centered on ablation of the LES. Large-diameter balloon pneumatic dilation (PD) and laparoscopic Heller myotomy (LHM) are the current mainstays in achalasia therapy with botulinum toxin injection (BTI) reserved for the frail elderly. A recent pivotal article noted equivalent results between PD and LHM with Dor's fundoplication at 43 mo post-therapy as noted by LES pressure measurements and dysphagia scores[1]. LHM is still considered the best therapy for achalasia because of its durable response and the possibility of perforation with PD[2]. A systematic review and meta-analysis noted LHM to yield superior and more durable results than PD and BTI[3]. End stage achalasia with a hugely dilated esophagus may necessitate partial esophagectomy (recently performed *via* laparoscopy)[4].

HISTORICAL BACKGROUND

Ortega described a case series of 17 achalasia patients in 1981 described a direct trans-mucosal lower esophageal sphincter myotomy and good clinical, radiologic and manometric results; there were no confirmatory work perhaps related to complications such as perforation and mediastinitis[5]. Natural orifice transluminal endoscopic surgery (NOTES) came to the fore in 2004 and there has been a push to design endoscopic less traumatic alternatives to traditional transcutaneous surgical interventions. Thus the concept of a submucosal tunnel closed by a mucosal flap with access to the mediastinum or the peritoneum was formulated[6]. Interventions on the esophageal muscularis could be performed at a distance from a mucosal entry point, which is then closed at the procedure termination. The concept was implicated in 2007 to perform a LES myotomy in a porcine survival model[7]. In 2008, Inoue used the technique of submucosal tunneling to perform the first human endoscopic LES myotomy for achalasia and coined the term POEM for per oral endoscopic myotomy[8]. Our group performed the first human POEM outside Japan in 2009[9]. There is a rapidly increasing international experience with over fifty centers now performing POEM. An international survey (IPOEMS) was performed describing the global POEM experience through July 2012[10].

INDICATIONS AND CONTRAINDICATIONS

Patients considered for POEM therapy must have a high-quality (preferably high resolution) esophageal manometry to define their specific motor abnormality as this will dictate both their eligibility for POEM and specifics of the procedure. POEM is performed predominantly for achalasia, but this technique has also been successfully applied in diffuse esophageal spasm, nutcracker and jackhammer esophagus[11,12]. The international survey (IPOEMS) revealed that 28% of the 841 reported POEMs performed by the 16 reporting centers, were performed for hypertensive esophageal motor disorders disorders including DES, hypertensive LES, nutcracker and jackhammer esophagus[10]. The consensus from IPOEMS and other studies is that POEM has significant efficacy in nutcracker esophagus, hypertensive LES, DES and type Ⅲ (spastic) achalasia[10-13]. POEM seems to be more helpful in diminishing dysphagia than the chest pain associated with these disorders[13]. POEM may be ideally suited in the treatment of patients with hypertensive esophageal motor disorders other than achalasia because in those disorders often a longer myotomy is required than can be achieved *via* the laparoscopic approach.

Experienced POEM operators can also now successfully treat patients with prior Heller myotomy and previous endoscopic intervention including botulinum injection and pneumatic dilation[14,15]. Forty-three percent of subjects in the IPOEMS database had prior intervention (BTI, PD, LHM)[10]. The consensus was that that the cases were more technically challenging, but there was similar good efficacy with few adverse events as the total subjects group. Subsequent smaller reported studies are consistent with these findings[14,15].

POEM has been performed throughout the age spectrum[10,16]. Most experienced operators will consider POEM in the gamut of achalasia subtypes, achalasia with "sigmoid" esophagus and medically fragile patients. POEM has been performed in post-gastric bypass patients with achalasia[17]. POEM contraindications by consensus include severe pulmonary disease, esophageal irradiation, esophageal malignancy, bleeding disorders including coagulopathy and recent esophageal surgery or endoscopic intervention including endoscopic mucosal resection and endoscopic submucosal dissection (ESD)[10].

POEM TECHNIQUE

POEM was developed from a technique devised to access the mediastinum in NOTES[6]. POEM features the creation of a submucosal tunnel enabling the LES myotomy to be performed away from the mucosal entry site. The procedure requires equipment employed in ESD with capability for dissection, cutting, coagulation and infusion of volume-expanders within the submucosal space. POEM is performed with general anesthesia with the patient usually in the supine position. Carbon dioxide is used for insufflation utilizing a high-definition forward-viewing gastroscope with accessory water jet for irrigation with an attached clear cap. The esophageal submucosal space is expanded with injection of an indigo carmine-saline mixture[18]. The submucosal tunnel is typically initiated 10-15 cm above the gastroesophageal junction (GEJ).

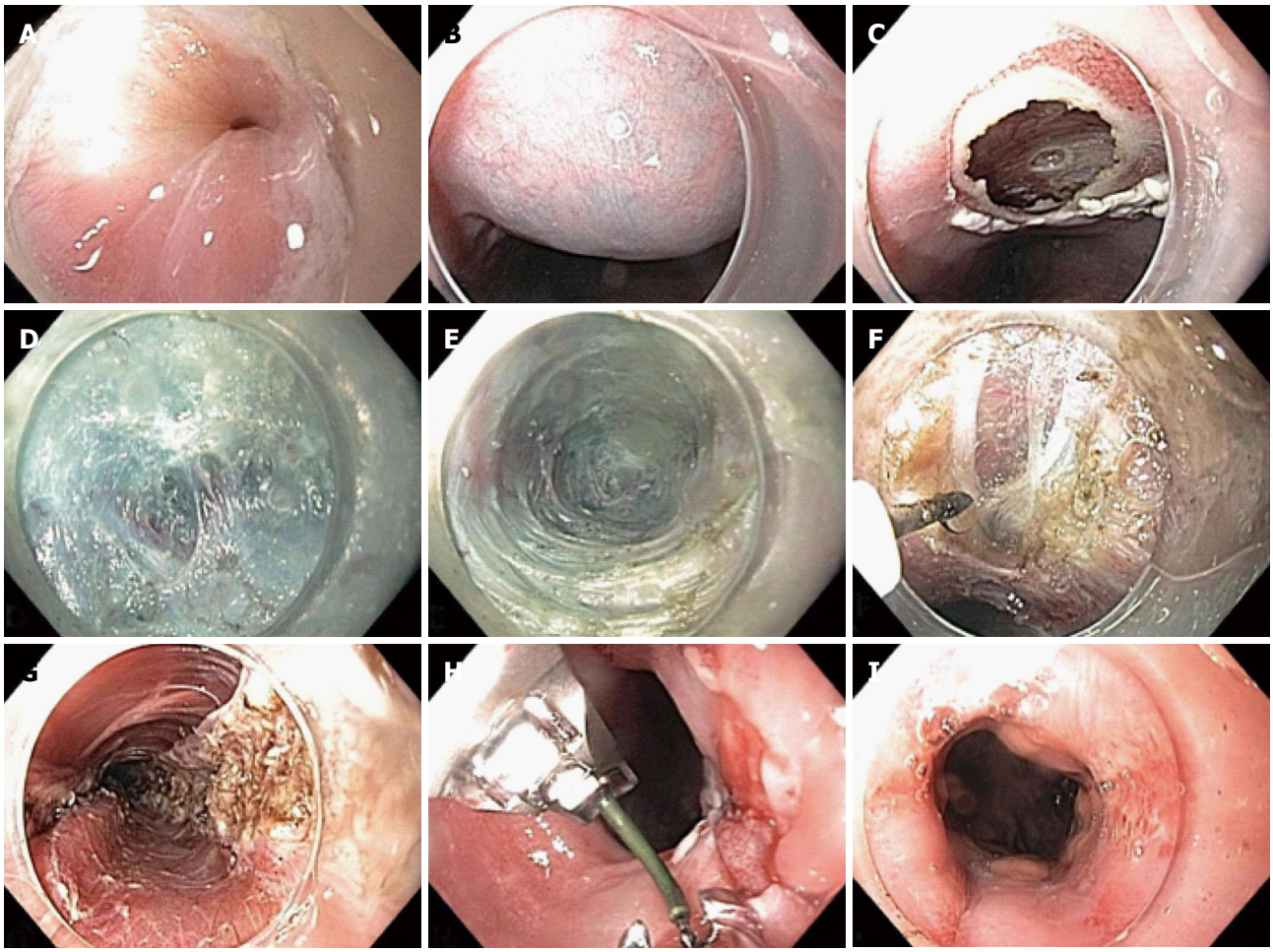

Figure 1 Per-oral endoscopic myotomy technique. A: Prior to per-oral endoscopic myotomy (POEM), there is evidence of a tightly puckered lower esophageal sphincter (LES); B: Submucosal injection is performed with saline stained with indigo carmine; C: Mucosotomy is performed along the right anterior wall of the esophagus; D: Submucosal dissection is performed with hybrid knife; E: Submucosal tunnel is extended into the gastric cardia; F: Myotomy is intiated 2 cm below site of mucosotomy; G: Final full thickness myotomy is seen as endoscope is withdrawn from the submucosal tunnel; H: Mucosotomy is closed with an endoscopic suturing device; I: After POEM, the LES appears patulous.

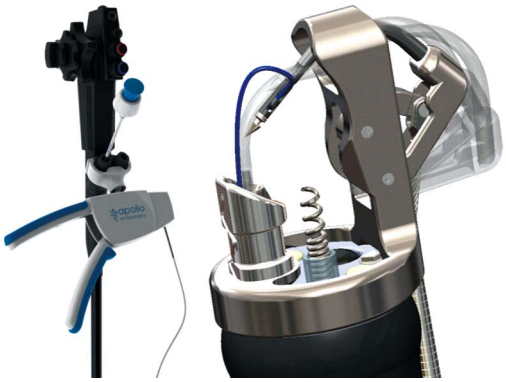

Figure 2 OverStitch endoscopic suturing system (Courtesy of Apollo Endosurgery, Austin Texas).

The submucosal space is entered by the gastroscope after a small electrosurgical cut and subsequently a submucosal tunnel is dissected using ESD technique for creation of a conduit to the GEJ and the lower esophageal sphincter. The submucosal tunnel is extended into the gastric cardia 2-3 cm distal to the GEJ. Delineation of the GEJ while in the tunnel is done in a variety of ways including monitoring endoscope insertion length, visually noting changes in tunnel diameter and vascularity, tactile feedback and even transillumination viewed by a second endoscope[19]. Then the myotomy is performed starting 2-3 centimeters distal to the mucosal entry point. The final critical step is closure of the tunnel at the mucosal entry point. Figure 1 demonstrates the critical steps in the POEM technique. We have presented a detailed explanation of the technique in video format elsewhere (*VJGIEN*, 2013)[20]. Patients typically have a post-procedural contrast study to exclude complications such as perforations and may be discharged the following day if clinically well including ingestion of clear liquids. Antibiotics are given during the POEM and continued for several days after discharge.

TECHNIQUE VARIATIONS

Individual POEM operators have generally evolved in

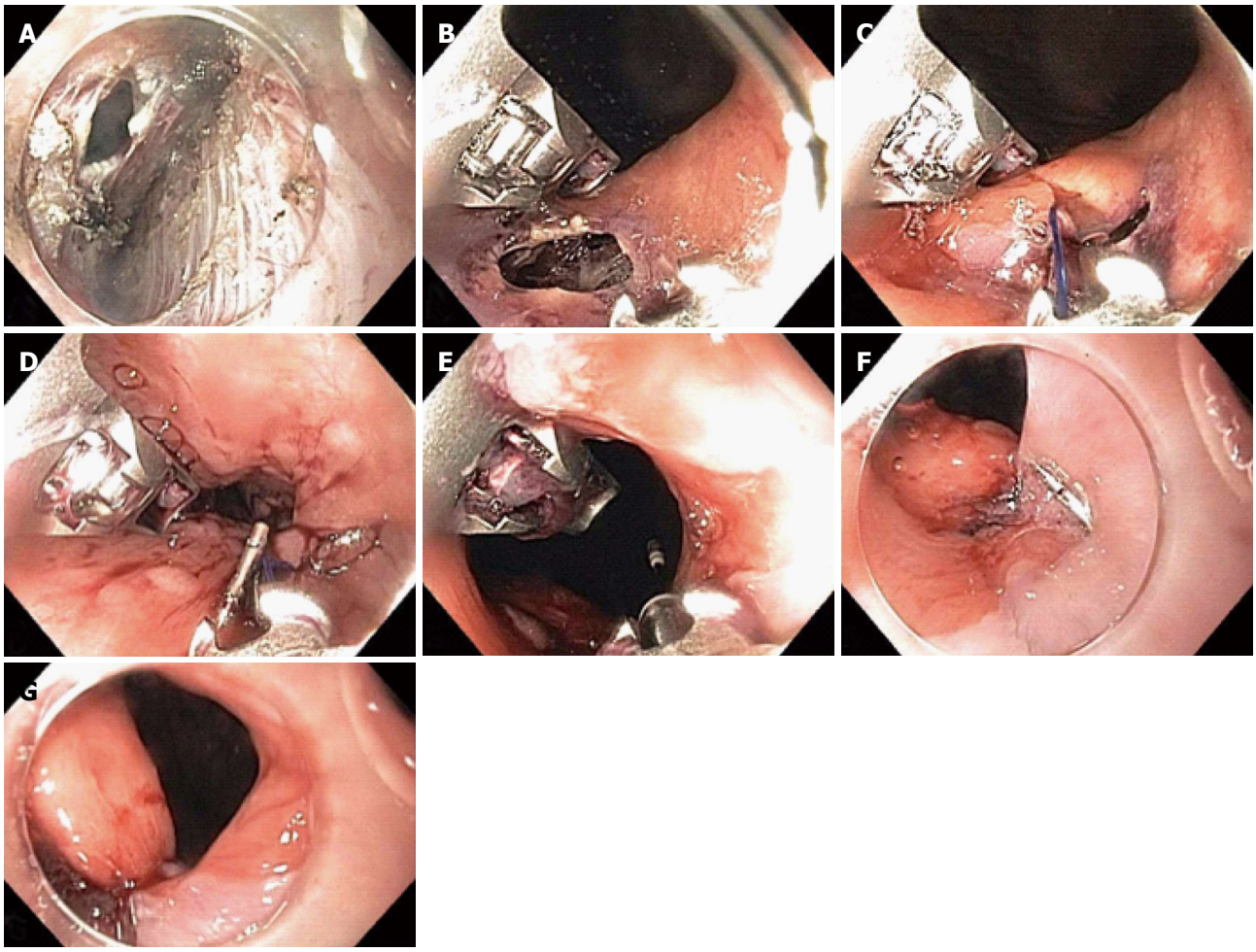

Figure 3 Closure of gastroesophageal junction mucosal perforation with endoscopic suturing device. A: Inadvertent mucosal perforation at the gastroesophageal junction (GEJ) seen within the submucosal tunnel; B: Inadvertent mucosal perforation at the GEJ seen endoluminally; C: Endoscopic suture-initial "bite"; D: Suture closure; E: Cinch T-tag is deployed; F: Endoscopic suturing achieved secure closure of the perforation; G: Patulous lower esophageal sphincter.

their techniques. The Shanghai group has converted to carbon dioxide after frequent serious complications with room air, and this group touts the hybrid (inject/cautery) ESD knife for faster dissection during POEM[21,22]. Inoue prefers the triangular tip knife and initially performed more modest length circular muscle myotomies[16]. Our group initially employed balloon dilation in tunnel creation (lack of ESD knives in the US before 2011), but now electrocautery dissection is exclusively used and we are more comfortable with extended length circular and longitudinal full-thickness muscle dissection[9,23]. Most centers employ clips for closure of the mucosal defect at the tunnel initiation. We have used an endoscopic suture device (OverStitch™ Endoscopic Suturing System; Apollo Endosurgery Austin, Texas) (Figure 2) for our recent cases and this is also invaluable for inadvertent mucosal tunnel perforation[24]. Figure 3 demonstrates a mucosal flap injury at the GEJ closed effectively with the endoscopic suturing device without concern for delayed leak in the patient. In our series when we compared the last 25 consecutive mucosotomy closures using endoclips and endoscopic suturing, there was no statistically significant difference in mean closure time (clips 8.8 min *vs* suture 10.1 min, $P = 0.1$), complications or mean cost (clips \$915.84 *vs* suturing \$818, $P = 0.2$) (unpublished data) Closure has also been described with an over-the-scope clip and fibrin glue[25,26].

There are POEM controversies regarding the orientation and extent of LES dissection. The human LES has several components that include a weaker clasp (circular) fiber part on the lesser curvature of the stomach centered at 2 o'clock (using the convention of 12 o'clock being the most anterior point) and a sling (oblique) fiber part on the left posterior lateral wall of the LES at 7 o' clock and draping over the anterior and posterior walls at 5 and 11 o'clock respectively[27]. These sling fibers are a significant barrier to reflux. Laparoscopic Heller Myotomy is often performed anteriorly at 11-12 o'clock and thus partially transects the sling fibers at 11 o'clock and thus GERD is common after LHM. Currently, most POEM operators reportedly perform myotomy at 2 o' clock, which may theoretically minimize post-procedure reflux, but be less efficacious as LES disruption is the main goal in achalasia surgery. We and others (Shanghai group) that employ a predominant 5 o'clock position for the myotomy may have less dysphagia because of sling

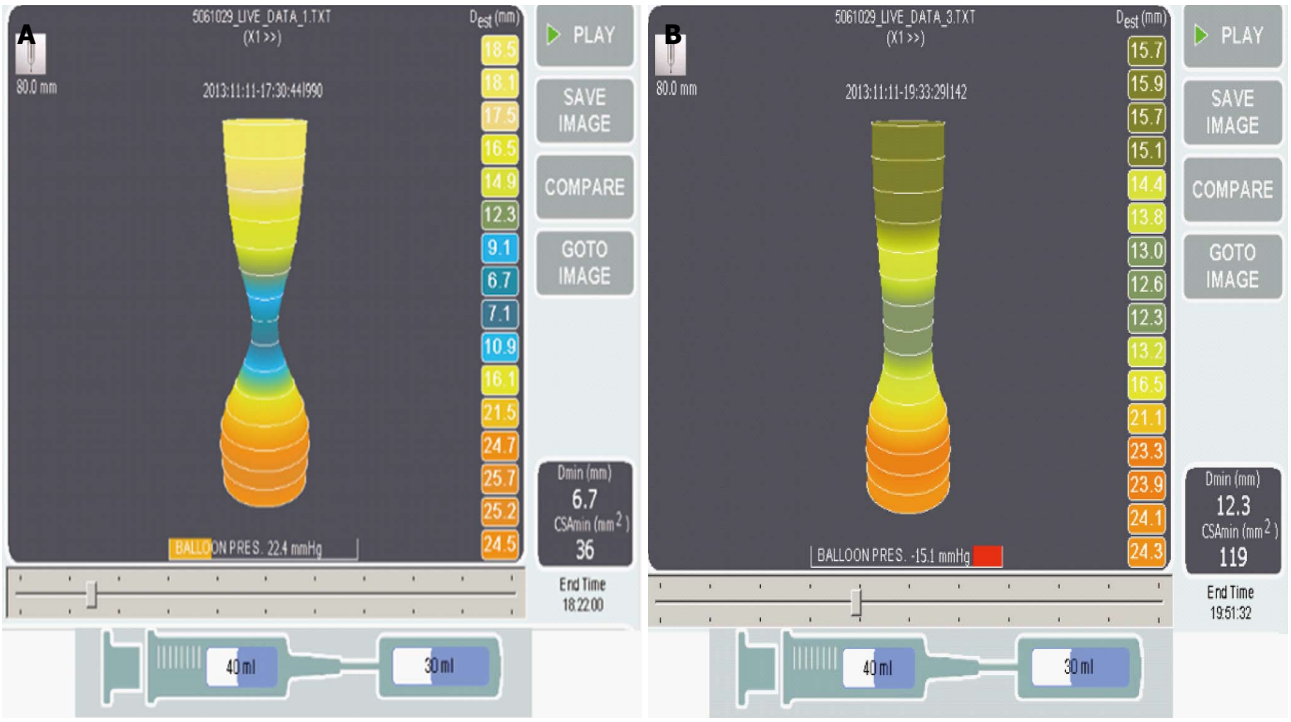

Figure 4 EndoFLIP Images before and after per-oral endoscopic myotomy. Seventy-seven years old man with achalasia for 4 years, prior Botox × 1, esophageal diameter of 5 cm, non-sigmoid, underwent per-oral endoscopic myotomy (POEM), 8 cm posterior myotomy. A: EndoFLIP performed immediately prior to POEM; B: immediately after POEM at 30 mL balloon volume shows an excellent response with increase of the minimal diameter at the gastroesophageal junction (GEJ) (Dmin) from 6.7 to 12.3 cm and increase in cross-sectional diameter at the GEJ (cross-sectional area) from 36 to 119 mm^2.

fiber resection but could theoretically have more GERD. We believe most post-POEM patients with GERD will do well with medical therapy. A lesser controversy with a similar theme is whether to dissect the weaker longitudinal muscle component of the LES along with the LES circular muscle. Additional resection of this longitudinal muscle may promote post-POEM reflux, but a study on this issue found no difference between POEM patients who had the additional resection and those that did not in terms of dysphagia relief and GERD[28].

ENDOFLIP

The adequacy of the POEM can be grossly assessed by visualization and passage of the gastroscope. Our group and other assess the esophagogastric junction distensibility quantitatively with the EndoFLIP system intraoperatively to confirm that an adequate myotomy has been performed[29]. The endoluminal functional lumen imaging probe (EndoFLIP®)(Crospon Ltd., Galway, Ireland) device is a balloon-tipped catheter that measures shape and compliance of the GEJ using impedance planimetry. It determines minimal luminal diameter and cross-sectional area (CSA) at the level of the LES. Real time quantitative assessment of GEJ distensibility can be calculated by dividing CSA by the balloon pressure that is also recorded by the device. This may assist in confirming that adequate myotomy has been achieved and possibly predict degree of dysphagia relief. A recent study by Rohof and colleagues demonstrated that GEJ distensibility more closely correlated with symptomatic recurrence after interventions for achalasia than manometry[30]. In our series, there was a patient who required additional LES dissection in another plane based on this data[23]. Using EndoFLIP intraoperative assessment in 43 POEMs at a 30 mL balloon inflation, there was a mean 3.5 fold increase in GEJ distensibility[31]. Figure 4 demonstrates nearly a two fold increase in Dmin diameter as well as three fold increase in cross-sectional area after myotomy. In a small but provocative study, intraoperative Endoflip measurements suggested that LHM requires more of a proximal esophageal myotomy to reduce GEJ distensibility than POEM[32].

EFFICACY AND SAFETY

The global results of POEM have been superb with dysphagia efficacy (using Eckardt score) in > 90% of subjects and no reported mortality in > 1000 patients[10,13,31,33-40] (Table 1). More limited data from these studies and smaller ones suggest significant diminution of LES pressure after POEM. Only a small number of studies have reported efficacy based on objective assessment of esophageal emptying by timed barium esophagram. However, their results confirm the high efficacy of the procedure (Table 1). We have performed > 130 cases with similar efficacy and safety.

GERD as defined by symptoms and available pH and endoscopy data is the most prevalent adverse event after POEM, though prevalence varied widely[10,13,23,31,35-38,40]. In the European multicenter POEM study, esophagitis

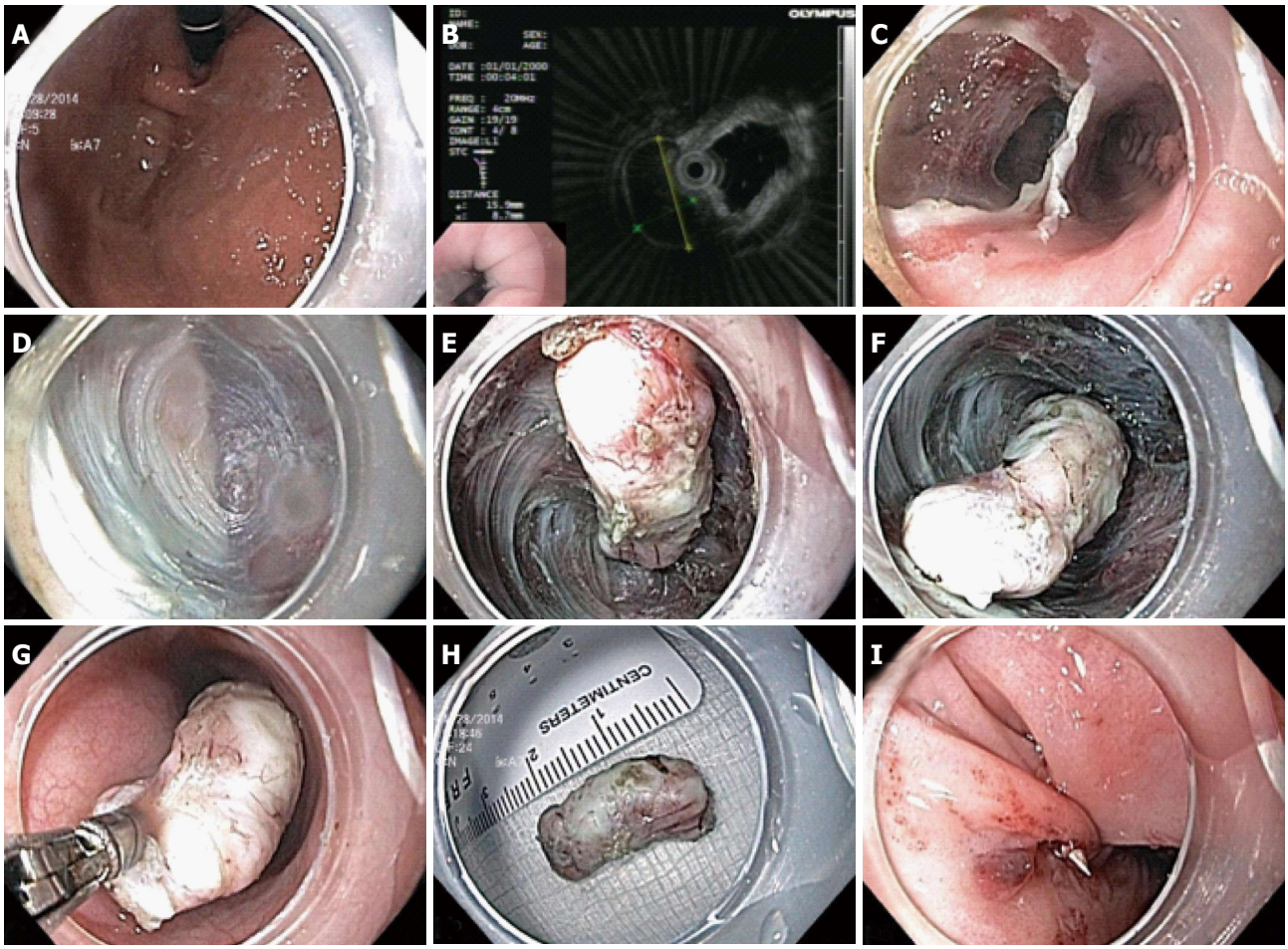

Figure 5 Submucosal tunneling endoscopic resection technique. A: Gastroesophageal junction lesion seen on retroflexion during endoscopy; B: Endoscopic ultrasound probe demonstrates hypoechoic muscularis propria based lesion; C: Creation of submucosal tunnel parallel to esophageal lumen; D: Endoscopic submucosal dissection (ESD) with submucosal tunnel; E-F: Freeing of submucosal lesion *via* ESD; G: Removal of submucosal lesion from tunnel with biopsy forceps; H: 2.5 cm leiomyoma; I: Endoscopic sutured closure of mucosal entrance to tunnel.

was in noted in 42% of subjects though was usually mild[38]. A small study suggested almost 40% of POEM subjects had abnormal acid exposure on pH testing, but this was similar to LHM subjects[41]. Insufflation related complications are less common with CO_2 insufflation[42]. Damage to overlying esophageal mucosa seems to be the common technical concern for the POEM operator. Modest rents can usually be remedied with clips or endoscopic suturing[24]. Bleeding in the tunnel is unusual but may require reentry for hemostasis[26].

Comparison to laparoscopic Heller myotomy

There is a lack of long-term efficacy of POEM generally and in relation to LHM or PD. There are no randomized controlled trials comparing POEM to LHM. One prospective series compared 18 POEM and 21 LHM subjects and noted no difference in operative time, myotomy length, relief of dysphagia or complications (each group had one perforation), but the POEM subjects had less postoperative pain and quicker return to work[43]. However, another group comparing 34 POEM to 64 LHM subjects found the POEM group to have less operative time, lower postoperative Eckardt scores, lower LES pressures, less length of stay and less dysphagia at six month follow-up[41]. A third series comparing 70 POEM and 110 LHM subjects found lower Eckardt and LES scores in the POEM group with no significant difference in erosive esophagitis[44]. More such comparative studies with longer follow-up are expected.

FUTURE PERSPECTIVES

There is current intense investigation in comparing POEM and LHM for achalasia, but long term data for POEM is lacking[13,41]. The available comparison bodes well for POEM with relative shorter operative times and hospital stays, lower cost and greater patient satisfaction[36]. Diminishing efficacy of POEM with time is a concern, but more studies are needed[38]. There is controversy regarding POEM operator background and training as well as credentialing. The apparent success of POEM has fueled some of this debate. Most POEM operators in the US and the world are surgeons[10]. Yet our group and others feature gastroenterologists with an impressive track record[23]. The Inoue group feels that extensive animal model practice in paramount for POEM success[45]. Two

Table 1 Overview of peroral endoscopic myotomy efficacy and gastro-esophageal reflux disease data

Location primary investigator	Patients (*n*)	Mean age (yr)	Eckardt score (pre/post)	LES pressure (pre/post) (mmHg)	Follow-up (mo)	Timed barium esophagram	Efficacy	Objective GERD evidence *n* (%)
Costamagna *et al*[34], Rome, Italy	11	41 (23-68)	7.1/1.1	45.1/16.9	3		100%	
Swanstrom *et al*[13], Portland, Oregon	18	59 (22-88)	6/0	45/16.8	6	Median emptying at 5 min 15/16: 80%-100% emptying 1/16 less than 80% emptying	94%	Esophagitis (Grade 1 Savory-Miller classification) 4/14 (28) +pH study 6/13 (46)
Chiu *et al*[35], Hong Kong, China	16	47 (22-87)	5.5/0	43.6/29.8	3		100%	+pH study 3/15 (20)
Hungness *et al*[36], Chicago, Illinois	18	38 (22-69)	7/1	19/9	63	Median height 1 min 7 cm (0-15 cm) 2 min 5 (0-13 cm) 5 min 0 (0-9 cm) (P < 0.001)	89%	Esophagitis Los Angeles (LA) class A 2/15 (13.3) B 2/15 (13.3) C 1/15 (6.7)
Minami *et al*[37], Nagasaki, Japan	28	52 (19-84)	6.7/0.7	71.2/21	16[3]		100%	Esophagitis (11/28) 39.3% LA class M 2/28 A 7/28 B 1/28 C 1/28
Von Renteln *et al*[38], European MCT	70	45	6.9/1	27.6/8.9	12		82%	Esophagitis (42) LA class A 29.2% B 12.3%
Stavropoulos *et al*[31], Mineola, New York[1]	100	52 (17-93)	7.8/0.2	44.2/17.6	13.3[2]	Mean emptying at 5 min 31/42: 100% emptying 41/42: > 50% emptying	98%	Esophagitis 17/53 (32) +pH study 17/52 (33)
Onimaru *et al*[39], Yokohama, Japan[1]	300	45 (3-87)	6.13/1.33	27.3/13.4	12		98%	
Verlaan *et al*[40], Amsterdam, Netherlands	10	43	8/1	20.5/6.8	3	Median height 1 min 3.2 cm (IQR 0.5-6.5) (*P* = 0.005) 2 min 2.7 cm (IQR 0.4-5.2) (*P* = 0.005) 5 min 2.3 cm (IQR 0-3.2) (*P* = 0.005)	100%	Esophagitis (60%) LA class A 3/10 (30) B 3/10 (30)

[1]Abstract; [2]Mean; [3]Median, remainder are minimum follow-up. GERD: Gastroesophageal reflux disease; LES: Lower esophageal sphincter; IQR: Interquartile range.

American "learning curve" studies were small and yielded somewhat conflicting results[46,47].

POEM OFFSHOOTS: OTHER RAPIDLY GROWING APPLICATIONS OF THE SUBMUCOSAL TUNNEL

POEM was derived from earlier NOTES experiments[5], but currently POEM is more clinically advanced and more widely adopted than any other NOTES procedure. NOSCAR has recently completed a comprehensive "White Paper" on POEM as a demonstration of this procedure's prominence[48]. More currently relevant is that the success of POEM has spurred interest and development in ESD; especially in the US[49]. Arguably POEM has contributed more to ESD adoption in the US in the past few years than over 10 years of presentations and publications by Japanese and other Asian endoscopists. However, the impact of POEM extends even further. POEM offers a controlled standardized setting for gastroenterologists to familiarize themselves with previously taboo spaces such as the mediastinum and peritoneum and become adept in fundamentals of NOTES such as managing insufflation (capnoperitoneum, capnomediastinum) and achieving secure closure of full thickness breeches of the GI lumen. Thus, the success of POEM has inspired other NOTES offshoots such as submucosal tunneling endoscopic resection (STER) to achieve full-thickness en bloc resection of muscularis based subepithelial tumors (Figure 5). Guidelines recommend resection of many of these tumors when they are larger than 2-3 cm, particularly if they are suspected to be gastrointestinal stromal tumors or if they are causing symptoms, increasing in size on surveillance or have high risk features on biopsy, endoscopic ultrasound (EUS) or computed tomography.

Table 2 Characteristics of United States single center submucosal tunneling endoscopic resection series *n* (%)

Characteristics	Value
STER cases (*n*)	7
Time period	9/2013-4/2014
Mean age (yr)	52 (47-62)
Gender	3 males: 4 females
ASA classification	
Ⅱ	6 (86)
Ⅲ	1 (14)
Location of lesion	4 GE junction; 2 esophagus; 1 gastric cardia
General anesthesia	100%
Procedure time (min)	53 (23-80)
Closure technique	2 clips (29); 5 endoscopic suture (71)
Histopathology	6 leiomyoma (86); 1 GIST (2/50 hpf) (14)
Mean size (cm)	1.5 (1-2.7)
En bloc resection	100%
Length of hospital stay (d)	2.3 (1-4)
Follow-up	100% no recurrence
Complications	1 mucosotomy required clipping 1 stricture at submucosal tunnel site responded to balloon dilation at 4 wk

GE: Gastroesophageal; STER: Submucosal tunneling endoscopic resection; ASA: American Society of Anesthesiologists.

Surgery can be difficult requiring resection of large portions of an organ such as partial gastrectomy or esophagectomy even for small tumors, especially those located in the esophagus, GE junction, pylorus or other challenging locations. For low risk lesions ≤ 2 cm, often endoscopic/endosonographic life-long surveillance is pursued. This overall approach generates a large burden of surgery and endoscopy for subepithelial tumors (SETs) less than 5 cm, the majority of which represent low risk lesions. STER may allow minimally invasive resection of submucosal tumors less than 3-4 cm and is especially appropriate for tumors in challenging locations for the surgeon including the GE junction, esophagus and gastric cardia. It offers a less invasive approach to resection for tumors requiring resection. For small tumors that were destined for surveillance it offers definitive histologic diagnosis (which not infrequently eludes standard endoscopic sampling such as EUS-guided fine needle aspiration). Furthermore, by achieving en bloc resection of these small low risk tumors it allows definitive histologic confirmation (including mitotic rate) of their benign nature and eliminates the need for life-long surveillance. It is not surprising that the initial reports of this technique originated at the two most prominent Asian POEM centers. Inoue *et al*[50] from Yokohama, and Li *et al*[51] from Shanghai reported their respective initial successes utilizing submucosal tunneling to safely and effectively achieve endoscopic resection of muscularis propria based submucosal tumors in a small series of patients. The Shanghai group subsequently reported their series of 290 patients with follow-up for 4 years showing no residual tumor, local tumor recurrence or distant metastasis[52]. Our group performed the first STER in the US in September 2012. Our small series of patients also supports that STER is a safe and feasible method for removing muscularis propria based SETs[53] (Table 2).

The principle of the submucosal tunnel technique has been adapted to perform wide resections of mucosal lesions (early cancers) in the esophagus. In a series of five patients, en bloc resection of superficial esophageal cancer was achieved in all without evidence of recurrence or complications including dysphagia at follow-up between 3-13 mo[54]. In a larger series of 25 superficial esophageal cancers, 92% *en bloc* resection was achieved with two patients having intramucosal carcinoma recurrence requiring further treatment at mean follow-up 22 mo[55].

There is a report from a small series employing submucosal tunnel endoscopy under conscious sedation to safely and effectively explore the peritoneal and thoracic cavities through transgastric peritoneoscopy[56].

Lastly, peroral pyloromyotomy (POP) using a tunneled approach very similar to that used to cut the lower esophageal sphincter in POEM, has been proposed as a potential therapy for gastroparesis[57]. Studies are in progress at a number of centers. If POP proves effective for even a subset of gastroparesis patients, it may have a large impact on the therapy of this challenging disease.

CONCLUSION

In conclusion, POEM has the potential to be the preferred modality for achalasia and related esophageal motor disorders when personnel and logistics allow. Many questions remain regarding POEM, but its future is bright. The impact of POEM is extending far beyond the narrow domain of esophageal motility disorders. As the first successful clinical application of submucosal endoscopy it is now spawning many other NOTES interventions utilizing a submucosal tunnel approach.

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REVIEW

Pathogenesis of alcoholic liver disease: Role of oxidative metabolism

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Abstract

Alcohol consumption is a predominant etiological factor in the pathogenesis of chronic liver diseases, resulting in fatty liver, alcoholic hepatitis, fibrosis/cirrhosis, and hepatocellular carcinoma (HCC). Although the pathogenesis of alcoholic liver disease (ALD) involves complex and still unclear biological processes, the oxidative metabolites of ethanol such as acetaldehyde and reactive oxygen species (ROS) play a preeminent role in the clinical and pathological spectrum of ALD. Ethanol oxidative metabolism influences intracellular signaling pathways and deranges the transcriptional control of several genes, leading to fat accumulation, fibrogenesis and activation of innate and adaptive immunity. Acetaldehyde is known to be toxic to the liver and alters lipid homeostasis, decreasing peroxisome proliferator-activated receptors and increasing sterol regulatory element binding protein activity *via* an AMP-activated protein kinase (AMPK)-dependent mechanism. AMPK activation by ROS modulates autophagy, which has an important role in removing lipid droplets. Acetaldehyde and aldehydes generated from lipid peroxidation induce collagen synthesis by their ability to form protein adducts that activate transforming-growth-factor-β-dependent and independent profibrogenic pathways in activated hepatic stellate cells (HSCs). Furthermore, activation of innate and adaptive immunity in response to ethanol metabolism plays a key role in the development and progression of ALD. Acetaldehyde alters the intestinal barrier and promote lipopolysaccharide (LPS) translocation by disrupting tight and adherent junctions in human colonic mucosa. Acetaldehyde and LPS induce Kupffer cells to release ROS and proinflammatory cytokines and chemokines that contribute to neutrophils infiltration. In addition, alcohol consumption inhibits natural killer cells that are cytotoxic to HSCs and thus have an important antifibrotic function in the liver. Ethanol metabolism may also interfere with cell-mediated adaptive immunity by impairing proteasome function in macrophages and dendritic cells, and consequently alters allogenic antigen presentation. Finally, acetaldehyde and ROS have a role in alcohol-related carcinogenesis because they can form DNA adducts that are prone to mutagenesis, and they interfere with methylation, synthesis and repair of DNA, thereby increasing HCC susceptibility.

Key words: Alcohol metabolism; Acetaldehyde; Reactive oxygen species; Alcoholic liver disease; Protein adducts; Hepatic stellate cells; Liver fibrosis; CYP2E1

Core tip: The goal of this article is to review the mechanisms of alcohol-mediated toxicity in parenchymal and non-parenchymal cells of the liver. Specifically, we highlight the effect of oxidative ethanol metabolites such as acetaldehyde and reactive oxygen species in the development of fat accumulation, fibrosis and deranged immune response.

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the major cause of morbidity and mortality worldwide and its clinical spectrum includes steatosis, fibrosis, alcoholic hepatitis (AH), cirrhosis, and hepatocellular carcinoma (HCC)[1]. Multiple factors (sex, obesity and genetic) are involved in the progression of ALD but how these aspects influence the clinical outcome remain unclear. More than 90% of heavy drinkers develop fatty accumulation but only 30% of alcoholics develop severe forms of ALD. Ethanol and the products of its metabolism have toxic effects on the liver and in recent decades, significant progress has been made in understanding the molecular mechanisms by which ethanol oxidative metabolism contributes to the pathogenesis of ALD[2]. Ethanol oxidation to acetate is a two-step process carried out by the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). These enzymes use NAD^+ as a cofactor (Figure 1).

ADH first oxidizes ethanol to acetaldehyde, which is then further oxidized to acetate by ALDH. In humans, there are at least eight isoenzymes of ADH and four of ALDH. ADH is a family of cytosolic enzymes mainly present in the liver but also in the gastrointestinal tract, kidney, nasal mucosa, testis and uterus. They are classified into five classes (ADH1-5) that differ in their structural and kinetic characteristics. ADH1 plays the major role in the metabolism of ethanol in the liver[3-7]. As a result of its electrophilic nature, acetaldehyde[8] can bind and form covalent chemical adducts with proteins, lipids and DNA[9-13]. These adducts are broadly pathogenic because they alter cell homeostasis, changing protein structure[11,12,14,15] and promoting DNA damage and mutation.

ADH and ALDH reactions lead to an accumulation of NADH and the consequent reduction of NAD^+/NADH ratio that has a significant effect on important biochemical pathways such as glycolysis, citric acid cycle, fatty acid oxidation, and glucogenesis. NADH is mainly reoxidized to NAD^+ by the mitochondrial electron transfer chain[16,17]. During the electrons transfer to oxygen, different reactive oxygen species (ROS) such as superoxide anion ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\cdot}$) are formed[16]. These species are unstable and rapidly react with additional electrons and protons. Although most of these ROS are converted to water before they can damage cells[18], a small proportion can generate toxic effects as lipid peroxidation, enzymes inactivation, DNA mutations, and destruction of cell membranes[19-21].

Another metabolic system involved in ethanol metabolism is the microsomal ethanol oxidizing system (MEOS) constituted by the cytochrome P450 (CYP) enzymes. These proteins are a superfamily of heme enzymes involved in oxidation of numerous endogenous substrate such as steroids, fatty acid and xenobiotics[22]. They catalyze many different reactions, such as monooxygenation, peroxidation, dealkylation, epoxidation, and dehalogenation in order to convert different chemical molecules in more polar metabolites to be excreted. An ethanol-inducible form of P450[23] catalyzes ethanol oxidation at rates much higher than other CYP enzymes. In physiological conditions only a small amount of ethanol, about 10%, is oxidized to acetaldehyde by CYP2E1[24] but during chronic alcohol abuse there is induction of the microsomal system[25,26], and an increase in CYP2E1 protein expression. The increase in CYP2E1 during chronic ethanol intake is correlated with a decrease in proteasomal degradation, which increases CYP2E1 protein stability[27,28]. Multiple factors such as insulin, acetone, leptin, adiponectin and cytokines regulate CYP2E1 mRNA and protein expression[29] and CYP2E1 expression levels depend on nutritional and metabolic conditions. For example, genetic obese mice or high-fat-diet-fed rats have high levels of CYP2E1[30,31]. Furthermore, increased CYP2E1[32] is found in diabetes, probably due to insulin post-transcriptional modulation[33,34]. CYP2E1 catalyzes the oxidation of ethanol to acetaldehyde and it can catalyze the oxidation of the latter to acetate[35] but this reaction is disadvantageous in the presence of ethanol[36]. The catalytic reaction of CYP2E1 generates a significant amounts of ROS, such as $O_2^{-}\cdot$, H_2O_2, $OH^{\cdot}$ and the hydroxyethyl radical (HER)[29,37].

H_2O_2 can react with metal ions to produce highly reactive $OH^{\cdot}$ radicals[37,38] and determine a broad range of adverse biological responses[37,39]. Lipid peroxidation is probably the most important reaction involved in alcohol-induced liver damage[40,41] by the formation of toxic aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which, similar to acetaldehyde are able to react with DNA to form exocyclic DNA adducts. DNA adducts such as N2-ethyldeoxyguanosine (N2-EtdG)[40] and 1,N(2)-propano-2'-deoxyguanosine (PdG) are detectable in livers of alcohol-exposed mice, and in alcohol-associated cancers[42] in humans. They generate DNA-protein and DNA interstrand crosslinks[12] and produce replication errors and mutations in oncogenes or oncosuppressor genes[43] with genotoxic, mutagenic and carcinogen effects[43]. Aldehydes generated by ethanol metabolism can also crossreact to form hybrid adducts. For example, MDA/acetaldehyde hybrid adducts (MAAs) potentiate carcinogenic effect of single adducts[10,44,45], thereby perpetuating their genotoxic effects. Autoantibodies against MMA were significantly elevated in sera of chronic alcohol-exposed animals[46] and in patients with ALD, and the titers are correlated with the severity of liver damage[11,47,48] and progression of liver fibrosis. Interestingly, adducts accumulate in perivenous regions both in alcohol-fed rats[49,50] and in the liver of alcoholics[51,52], overlapping with the distribution of fatty accumulation.

Peroxisomal catalase is an additional metabolic path-

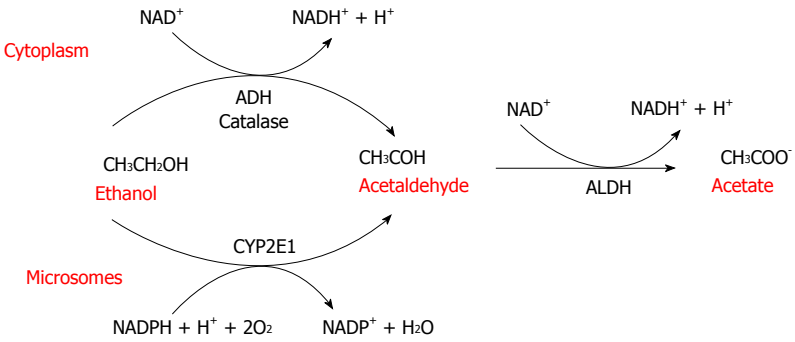

Figure 1 Alcohol metabolism. Alcohol dehydrogenase (ADH) is the main cytosolic enzyme that converts alcohol to acetaldehyde. The inducible microsomal enzyme also forms acetaldehyde. The toxic metabolite acetaldehyde is then further oxidized to acetate by the mitochondrial aldehyde dehydrogenase (ALDH).

way involved in ethanol oxidation. Catalase is a heme-containing enzyme that normally catalyzes the removal of H_2O_2 but it can catalyze the oxidation of alcohol to acetaldehyde. This pathway is not significant in the liver, but seems to be important in the brain. In fact acetaldehyde produced from catalase-dependent oxidation of ethanol seems to play a role in tolerance and alcohol addiction interfering with catecholamine neurotransmission[53-55].

MECHANISMS OF ALCOHOLIC FATTY LIVER

The earliest response of the liver to alcohol abuse is characterized by lipid accumulation in hepatocytes, which is a reversible condition but can progress to inflammation and fibrosis. The mechanism of triglyceride and fatty acid accumulation in the liver during alcohol consumption involves regulatory pathways that control lipid synthesis, oxidation and very-low density lipoprotein exportation. Short-term studies on isolated hepatocytes or perfused liver have shown that ethanol reduces the rate of β-oxidation and stimulates fatty acid uptake[56]. The increased production of reducing equivalents (NADH) from ethanol oxidation by ADH is believed to cause a shift in the cytosolic NADH/NAD^+ ratio, which in turn increased NADH/NAD^+ ratio in the mitochondria. Many of the enzymes of fatty acid oxidation are pyridine nucleotide dependent, thus, their activities are inhibited by NADH, resulting in reduced ability to oxidize fatty acids[57,58]. Although generation of reducing equivalents by ADH is sufficient to cause lipid accumulation[59], the finding that fat infiltration in the liver persists despite normalization of NADH/NAD^+ ratio, and that antioxidants prevent it in rats chronically fed alcohol, suggest that additional mechanisms are involved[60] (Figure 2). The role of peroxisome proliferator-activated receptors (PPARs) in fatty liver disease has been investigated in the past decade. These receptors are members of steroid/retinoid nuclear receptor superfamily of transcription factors[61,62]. PPARα regulates transcription of genes involved in the esterification and export of fatty acids and oxidizing them in the mitochondria, peroxisomes, and microsomes.

PPARα-null mice fed with Lieber-DeCarli diet exhibited hepatomegaly, macrovesicular steatosis, hepatocyte apoptosis, and hepatic fibrosis; all aspects resembling the pathological features of ALD[62], and suggesting that inhibition of PPARα transcriptional activity is implicated in fat accumulation. Ethanol metabolism, by way of acetaldehyde, interferes with the transcriptional activity of PPARα in hepatoma cells[62]. This effect is accompanied by a reduction in the ability of this receptor to bind its DNA consensus sequence, reflecting a possible covalent modification by acetaldehyde or changes in its phosphorylation state. Accordingly, chronic ethanol feeding in mice inhibited PPARα DNA binding activity and decreased PPARα target genes[63,64]. In mouse models of ALD, treatment with PPARα ligands such as WY14, 643 and clofibrate, restores receptor activity and significantly ameliorates fat accumulation and necroinflammation[63,64]. In addition, ethanol can also inhibit PPARα *via* upregulation of CYP2E1-derived oxidative stress[65].

Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors strictly correlated with PPARs and they control a set of enzymes involved in the synthesis of fatty acids and triglycerides. acetaldehyde produced from ethanol metabolism enhances the levels of SREBP-1 in hepatoma cells[66] and SREBP-1 protein levels are increased in animal models of alcohol-induce hepatic fat accumulation[66,67]. The role of SREBP-1 in alcoholic steatosis has been confirmed by several studies that couple the levels of this transcription factor with the ability to promote alcoholic fat accumulation by tumor necrosis factor (TNF)-α[68], circadian gene Per-1[69], early growth response (Egr)-1[70], epinephrine[71] and ER stress response[72]. In response to acute and chronic ethanol exposure, mitogen-activated protein kinase family members, including c-Jun N-terminal protein kinase (JNK), are activated and JNK inhibitors blunt steatosis, reducing oxidative stress and blocking SREBP-1 expression in hepatoma cells[73]. Recent studies have demonstrated that phosphatidylinositol 3-kinase (PI3K)/AKT pathway activation is involved in acute ethanol-induced fatty liver in mice, and specifically inhibits the phosphorylation and degradation of SREBP-1[74]. SREBP-1 is also modulated by AMP-activated protein kinase (AMPK).

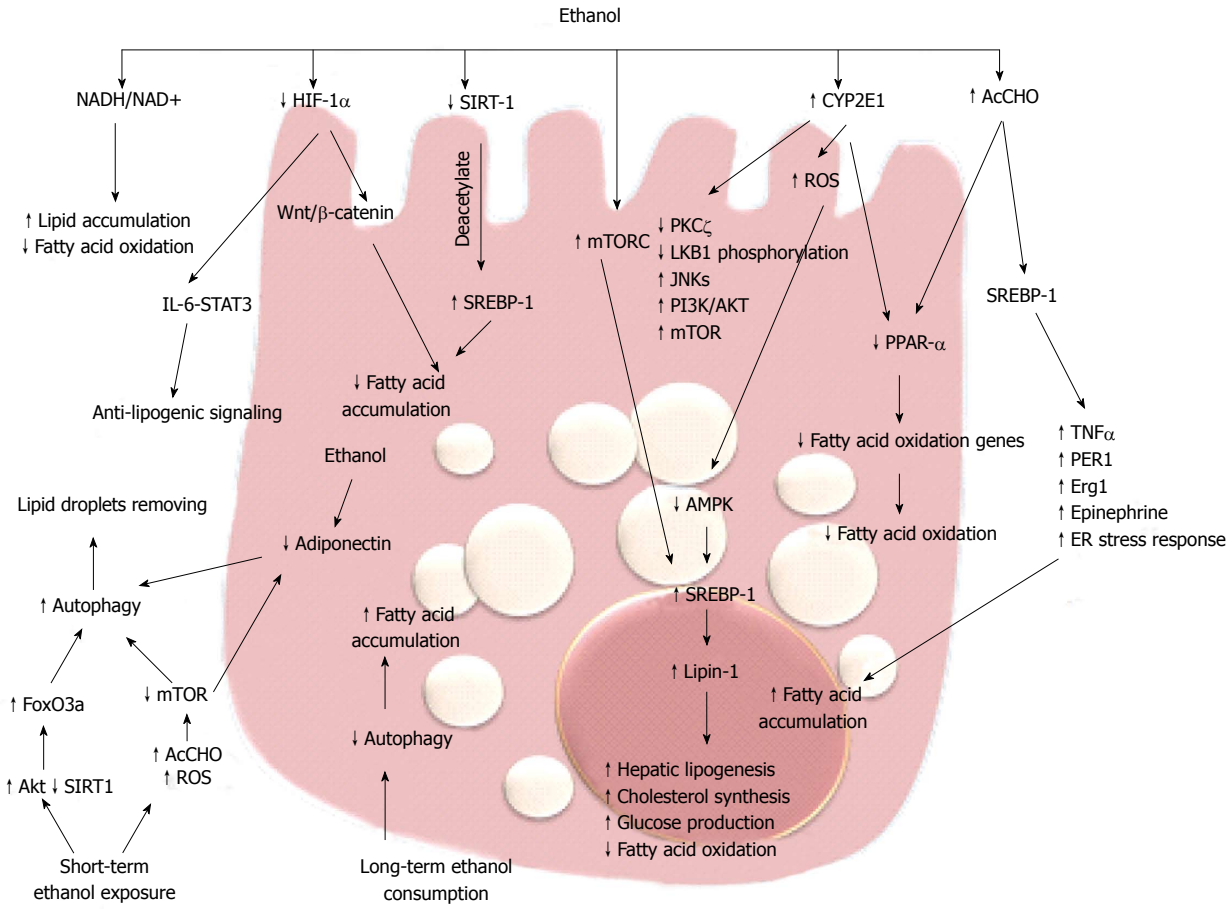

Figure 2 Molecular mechanisms of alcoholic fatty liver. Alcohol consumption *via* multiple pathways increases the expression of SREB-1 and downregulates PPAR-α, promoting fatty acid synthesis and impairing β-oxidation, thus resulting in fatty acid accumulation. Long-term ethanol consumption promotes fatty acid accumulation through decreased autophagy, while short-term ethanol exposure promotes autophagy and degradation of lipid droplets. HIF: Hypoxia inducible factor; ROS: Reactive oxygen species.

AMPK is a key player in the regulation of cellular energy homoeostasis by limiting anabolic pathways (to prevent further ATP consumption) and by facilitating catabolic pathways (to increase ATP generation). AMPK is a metabolic sensor by phosphorylation of enzymes involved in lipid metabolism. Chronic ethanol exposure inhibits AMPK activity in cultured rat hepatocytes through the inhibition of protein kinase (PK)Cζ and liver kinase (LK)B1 phosphorylation[75], and impaired AMPK activity was shown in hepatocytes isolated from rats fed with ethanol[76]. This inhibition plays a key role in the development of steatosis by the activation of hepatic lipogenesis, cholesterol synthesis, and glucose production in parallel with the decrease in fatty acid oxidation[74]. In rat hepatoma cells, overexpression of a constitutively active form of AMPK blocked the effect of ethanol, but in contrast, a dominant negative form augmented the effect through regulating SREBP-1[77]. Recent data have demonstrated that Lipin-1, a Mg^{2+} phosphatidate phosphatase involved in the biosynthesis of triacylglycerol and the transcriptional regulation of lipid homeostasis, is upregulated by ethanol through inhibition of AMPK and activation of SREBP-1[78]. Increased intracellular concentrations of ROS may represent a general mechanism for the enhancement of AMPK-mediated cellular adaptation, including the maintenance of redox homeostasis. AMPK activation by ROS can promote cell survival by inducing autophagy, mitochondrial biogenesis, and expression of genes involved in antioxidant defense.

Autophagy is a genetically programmed, evolutionarily conserved process of cellular catabolism that serves to maintain a balance among protein synthesis, degradation, and recycling. Autophagy implies degradation of damaged organelles and cellular protein in order to promote cell survival[79]. The mammalian target of rapamycin (mTOR) is a key regulator of autophagy. During deprivation of nutrients or other cause of cellular stress, there is inhibition of the mTOR/rapamycin pathway and consequent activation of autophagy in hepatocytes[80,81]. There are contrasting data regarding the effect of ethanol metabolism on autophagy. Long-term alcohol consumption inhibits autophagy[82] but one recent study has shown that ethanol metabolism upregulates autophagy in cultured hepatoma cells[83]. Short-term ethanol exposure activates autophagy by generating acetaldehyde and ROS and inhibiting mTOR. These data indicate that acute ethanol activation of autophagy could have a compensatory role that prevents development of steatosis during the

early stages of alcoholic liver injury[84]. Beyond mTOR, there are several other pathways involved in the induction of autophagy. Recently, Ni *et al*[84] demonstrated that *in vivo* and *in vitro* acute ethanol treatment activates nuclear translocation of forkhead box (Fox)O3a and expression of FoxO3a target genes. The authors suggest that the ethanol activation of FoxO3a could be mediated by Akt activation. In primary hepatocytes, expression of a dominant negative form of FoxO3a inhibits ethanol-induced, autophagy-related genes and improves ethanol-induced cell death, suggesting that FoxO3a is a key factor in regulating ethanol-induced autophagy and cell survival[85,86]. In addition, Sirtuin (SIRT)-1, the NAD^+-dependent protein deacetylase is indicated as an intermediary between autophagy and transcriptional regulation of lipid metabolism. In rat hepatoma cells expressing alcohol-metabolizing enzymes, ethanol reduces SIRT-1 expression and impairs SIRT-1-induced deacetylation of SERBP-1, leading to an increase in fatty acid synthesis[87]. The findings that the master regulator of autophagy mTOR complex 1 (mTORC1) regulates SERBP-1 by controlling the nuclear entry of lipin-1[88], and that adiponectin protects liver cells from ethanol-induced apoptosis *via* induction of autophagy[89,90], indicate that ethanol metabolism affects different metabolic targets of a complex transcriptional network that controls hepatic lipid homeostasis.

Recent intriguing data correlate ethanol-induced fat accumulation with the hypoxia-inducible factors (HIFs). HIFs are the master regulators of oxygen homeostasis and regulate the expression of many genes involved in glycolysis, glucose transport, and synthesis of inflammatory and proangiogenic cytokines[89-91]. The HIF-1α protein is rapidly degraded under normoxic conditions, whereas hypoxia enhances HIF-1α levels by inhibiting its degradation[92-94]. HIF-1α has been implicated in many models of liver injury[95] and it has been reported that feeding mice for 4 wk with the Lieber-DeCarli diet increases HIF-1α mRNA, protein, and DNA-binding activity in the liver. In addition, mice lacking HIF-1α in hepatocytes have a reduced hepatic steatosis and hypertriglyceridemia[96]. Conversely, Nishiyama *et al*[96], with a similar molecular technology, found that activation of HIF-1α suppresses ethanol-induced fatty liver. These discordant results between the two studies is difficult to explain, although recent data in a methionine- and choline-deficient diet model showed that upregulation of HIF-1α correlated with steatotic infiltration and activation of the Wnt/β-catenin signaling pathway[97,98]. Furthermore, the link between HIF-1α expression and the anti-lipogenic interleukin (IL)-6/signal transducer and activator of transcription (STAT)3 signaling[99-101] suggests that further studies are needed to clarify the role of hypoxia and the HIF pathway in alcoholic fatty liver.

MECHANISM OF ALCOHOL-INDUCED FIBROGENESIS

Hepatic fibrosis is a major histological feature associated with the progression of chronic liver disease to cirrhosis; it is characterized by increased deposition of components of the extracellular matrix (ECM), in particular fibrillar collagens types I and Ⅲ[102,103]. This process is associated with an upheaval of hepatic architecture, decreased number of endothelial cell fenestrations, and portal hypertension. The key event in hepatic fibrogenesis is hepatic stellate cell (HSC) activation. HSCs are one of the major sources of ECM in the liver and they have been identified as the precursor cell type mainly responsible for the development of liver fibrosis. Following liver injury, HSCs undergo activation that leads to the loss of the typical star-shape, fat-storing phenotype and acquisition of a myofibroblast-like phenotype consisting of increased cell proliferation, enhanced cytokine expression, and synthesis of ECM components[104,105]. Acetaldehyde is one of the main mediators of alcohol-induced fibrogenesis in the liver[106,107]. Early studies have shown that acetaldehyde can stimulate synthesis of fibrillar-forming collagens and structural glycoproteins of ECM in HSCs[108]. In addition, acetaldehyde promotes ECM remodeling by upregulation of the interstitial collagenase matrix metalloproteinase (MMP)-2 and downregulation of the fibrillary collagenase MMP-1, thus resulting in the substitution of the normal ECM components with a sclerotic matrix[109,110]. In human HSCs, acetaldehyde directly induces the transcription of the α_1(I) and α_2(I) procollagen genes by a PKC-dependent pathway, which is involved in rapid activation of activator protein (AP)-1 transcription factors[111] (Figure 3). In human HSCs, PKC phosphorylates p70s6k by a mechanism that involves extracellular signal-regulated kinase (ERK)1/2 and PI3K, and all these pathways lead to collagen α2(I) gene expression[112]. Both collagen α1(I) and α2(I) promoters have an acetaldehyde-responsive element (AcRE) that includes binding sites for different transcription factors including AP-1 and specificity protein (SP)-1. AP-1 activation is postulated to be involved in the acetaldehyde-induced expression of the basic transcription element binding protein (BTEB), which is able to transactivate the rat α_1(I) collagen promoter[113,114]. In addition, acetaldehyde modulates collagen α1(I) expression with a mechanism involving members of the CAAT/enhanced binding protein (C/EBP) family of transcription factors. Acetaldehyde increases DNA binding and transcriptional activity of C/EBPβ[115,116] with a mechanism that requires H_2O_2 production[117]. Similarly, acetaldehyde exerts its profibrogenic action by inhibition of the PPARγ transcriptional activity in HSCs[118]. PPARγ is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that is predominantly expressed in adipose tissue, where it has been shown to have a key role in adipogenesis and in regulation of insulin resistance[117]. Acetaldehyde inhibits PPARγ transcriptional activity in H_2O_2-dependent phosphorylation of the receptor[119-121]. Acetaldehyde stimulates H_2O_2 production that induces a signal transduction cascade that involves cAbl, PKCδ and ERK1/2. Acetaldehyde does not induce collagen

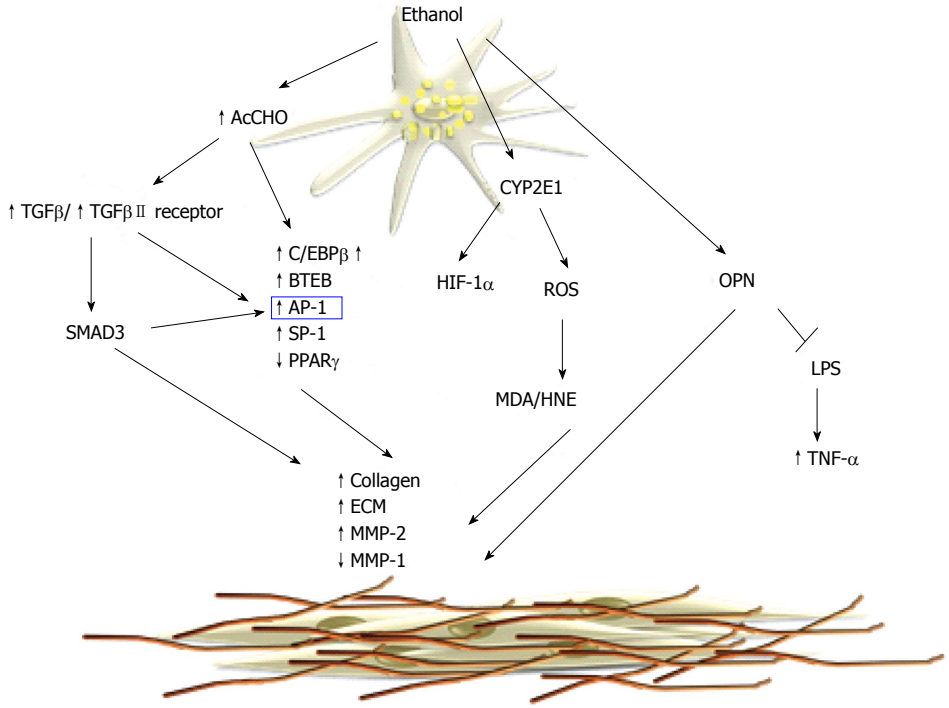

Figure 3 Molecular mechanisms of alcoholic fibrosis. Acetaldehyde causes increased synthesis of collagen and extracellular matrix (ECM) components through the activation of the transforming growth factor (TGF)-β/SMAD3 signaling pathway. The microsomal metabolism of ethanol leads to protein adduct formation that up-regulates collagen synthesis. MDA: Malondialdehyde; OPN: Osteopontin; LPS: Lipopolysaccharide; TNF-α: Tumor necrosis factor-α; MMP: Metalloproteinase; HNE: Hydroxynonenal; AP-1: Activator protein-1; SP-1: Specificity protein-1.

synthesis in quiescent HSCs[122] and it is not able to modulate PPARγ phosphorylation in these cells. The molecular events involved in the unresponsiveness of quiescent HSCs to fibrogenic stimuli[64], including acetaldehyde, remain speculative.

A different mechanism of acetaldehyde-induced fibrogenesis involved transforming growth factor (TGF)-β/small mother against decapentaplegic (SMAD) signaling. Acetaldehyde increases the secretion of TGF-β1 and induces TGF-β type Ⅱ receptor expression in HSCs[123]. In cultured human HSCs it has been shown that acetaldehyde upregulates collagen α1(I) mRNA expression *via* two distinct mechanisms[115,124]. An early TGF-β-independent response occurs within 3 h of acetaldehyde administration in human HSCs and selectively is correlated to SMAD3 phosphorylation[107]. On the contrary, longer acetaldehyde incubation induces a TGF-β-dependent late-phase response[125] characterized by induction of latent TGF-β1 secretion, as well as type Ⅱ TGF-β receptor expression[126]. Recently, acetaldehyde was shown to modulate β-catenin signaling[126] by a mechanism that inactivates nucleoredoxin (NXN) and release disheveled (DVL) from the NXN/DVL complex, leading to inactivation of glycogen synthase kinase (GSK)3B, and thereby blocks β-catenin phosphorylation and degradation. Thus, the stabilized β-catenin translocates to the nucleus where it upregulates fibrogenic genes[44,51,127,128]. It is still unclear whether the profibrogenic effects of acetaldehyde are mediated by its ability to form protein adducts. However, elevated levels of acetaldehyde-protein adducts correlate with the progression of liver fibrosis in alcoholic patients and animal experimental models[129]. Furthermore, neutrophil-derived ROS are able to induce lipid peroxidation and MDA/HNE protein adducts in HSCs, resulting in increased collagen synthesis[130].

The role of ROS and lipid peroxidation in hepatic fibrogenesis is well documented in cellular and animal models. CYP2E1-dependent generation of ROS increases collagen I protein synthesis in cocultures of hepatocytes and HSCs[131].

Recent work has shown that CYP2E1 activity correlates with ethanol-induced liver injury, lipid peroxidation, and collagen deposition[132]. CYP2E1 deletion effectively blocks ethanol-mediated lipid peroxidation and reduces liver injury, as shown in CYP2E$^{-/-}$ mice[133]. In contrast, transgenic mice overexpressing CYP2E1[134] enhance oxidant stress and hepatic fibrogenesis. Recently, it has been shown that that protein levels of HIF-1α and its downstream targets were elevated in the ethanol-fed CYP2E1-knock-in mice compared to the wild-type and CYP2E1 knockout mice, suggesting that CYP2E1 plays a role in ethanol-induced hypoxia. Angiogenesis is coupled with fibrogenesis during liver injury and HIF-1α contributes to CYP2E1-dependent collagen deposition and ECM remodeling. Recent studies have highlighted the role of osteopontin (OPN) in ALD and its correlation with hepatic fibrogenesis. OPN is a multifunctional protein, involved in different pathological conditions and it is associated with inflammation, autoimmunity, angiogenesis, fibrosis and cancer progression in various tissues. The OPN levels in the liver are correlated with fibrosis in patients with ALD[135]. OPN is profibrogenic by promoting HSC activation and ECM deposition *in vitro* and *in vivo*. Opn$^{-/-}$ mice have a significant delay in fibrosis resolu-

tion and a decreased expression of inflammatory cytokines[136]. Hepatic expression and serum levels of OPN are markedly increased in AH, compared to normal livers and other types of chronic liver diseases, and correlate with disease severity and short-term survival. Recent data show that OPN binds lipopolysaccharide (LPS) and protects against early alcohol-induced liver injury by blocking the TNF-α effects in the liver[137]. Furthermore, OPN is reported to be a downstream effector of the Hedgehog pathway, which modulates fibrosis and is involved in peculiar aspects of hepatic carcinogenesis[138].

ETHANOL OXIDATION AND ACTIVATION OF INNATE AND ADAPTIVE IMMUNITY

Innate immunity has a central role in the pathogenesis of ALD, and in recent decades significant progress has been made in understanding the molecular mechanism contributing to the alcohol-dependent activation of innate immunity and inflammation. Evidence indicates that alcohol consumption causes enteric dysbiosis and bacterial overgrowth[139,140] that leads to a significant increase in gut permeability and consequently high levels of LPS in the portal circulation[141-143]. Acetaldehyde contributes to alter intestinal barrier function and to promote endotoxin translocation by disrupting tight and adherens junctions in human colonic mucosa[144] *via* increasing tyrosine phosphorylation of occludin and E-cadherin. The mechanism of acetaldehyde-induced alteration of gut permeability remains unclear, although acute ethanol exposure upregulates miRNA-212 in enterocytes and this is correlated with zonula occludens-1 protein downregulation[145-150]. LPS interacts with toll-like receptor (TLR)4 to activate the MyD88-dependent and -independent (TRIF/IRF-3) signaling pathways and induces Kupffer cells to release ROS and an array of proinflammatory cytokines and chemokines including IL-1β, TNF-α, IL-6, IL-8, macrophage chemotactic protein (MCP)-1, and RANTES (regulated normal T cell expressed and secreted)[151]. ROS produced by Kupffer cells in response to endotoxin induces hepatic expression of TLR4[152,153], enhances transduction of TLR4-mediated signals through nuclear factor (NF)-κB, and activates mitogen-activated protein kinase (MAPK) pathways[154-157]. Several data indicate that TLR4 is the main player in the development and progression of ALD. TLR4 is also expressed in HSCs and endothelial cells, and regulates alcohol-induced proangiogenic and profibrogenic responses[158].

Kupffer cell activation contributes to intrahepatic recruitment and activation of granulocytes[159-161]. Acetaldehyde and LPS[162-164] stimulate parenchymal and non-parenchymal cells to produce IL-8, chemokine CXC ligand (CXCL)1 (Gro-α) and IL-17 that directly or indirectly contribute to neutrophil infiltration and severity of AH[165-167]. An alternative pathway that contributes to expression of inflammatory cytokines is the complement system. Ethanol oxidation activates C1q, C3 and C5 components that in turn stimulate Kupffer cells to produce TNF-α[168].

A recent study indicated that IL-1β has an important role in alcohol-induced steatohepatitis. IL-1β is a potent proinflammatory cytokine whose levels are increased in patients with ALD and correlated with oxidative stress. IL-1β maturation is dependent on caspase 1 in the multiprotein complex named inflammasome. *In vivo* intervention with a recombinant IL-1β receptor antagonist ameliorates inflammosome-dependent alcoholic steatohepatitis in mice, suggesting a potential role of IL-1 inhibition in the treatment of ALD[169-171].

On the contrary, convincing data demonstrate that activation of innate immunity also induces elevation of anti-inflammatory and hepatoprotective cytokines such as IL-10 and IL-6. These cytokines activate STAT3 in hepatocytes and Kupffer and endothelial cells, preventing alcohol-induced liver injury and inflammation[172]. As a matter of fact, the effect of ethanol oxidative metabolism on STAT3 in the liver is complex. STAT3 is a cell survival signal and protects against hepatocellular damage. STAT3 in the liver is significantly impaired in chronic alcoholic patients compared with other different liver diseases such as chronic hepatitis C and autoimmune disease. Furthermore, ethanol oxidation is correlated with suppression of natural killer (NK) cell function in the liver. NK cells have important antifibrotic function in chronic liver disease and several studies have indicated that during liver injury there is an elevated expression of NK cell ligands. Active crosstalk between HSCs and NK cells *via* TNF-related apoptosis-inducing ligand (TRAIL)-TRAIL receptor interactions and a consequent production of interferon (IFN)-γ results in NK cell cytotoxicity of HSCs, thereby limiting hepatic fibrogenesis[173]. Oxidative stress in chronic ethanol consumption induces increased levels of TGF-β and reduces IFN-γ signaling, blocking NK cell killing of activated HSC[174] (Figure 4). Cell-mediated adaptive immunity is another important aspect of host defense that can be altered by alcohol and its metabolites. The mechanisms by which alcohol triggers adaptive immunity are still incompletely characterized. Chronic alcohol ingestion can interfere with antigen presentation that is required to activate T and B cells and can impair dendritic cell differentiation[175-178]. Patients with AH have increased levels of circulating antibodies against modified protein adducts with HER and lipid-peroxidation-derived aldehydes, justifying the activation of the adaptive immune response[179,180]. HER and MDA antibodies have been detected in chronically ethanol-fed rats as well as in alcohol abusers, and they are associated with detection of peripheral blood $CD4^+$ T cells that are responsive to these adducts. The cytokines released by activated $CD4^+$ T cells can then further stimulate Kupffer cell activation, contributing to parenchymal injury, hepatic inflammation, and fibrogenesis.

Ethanol oxidation impairs proteasome function in macrophages through impairment of IFN-γ signaling, suppression of chymotrypsin-like proteasome activity, and the consequent composition of the immunoprotea-

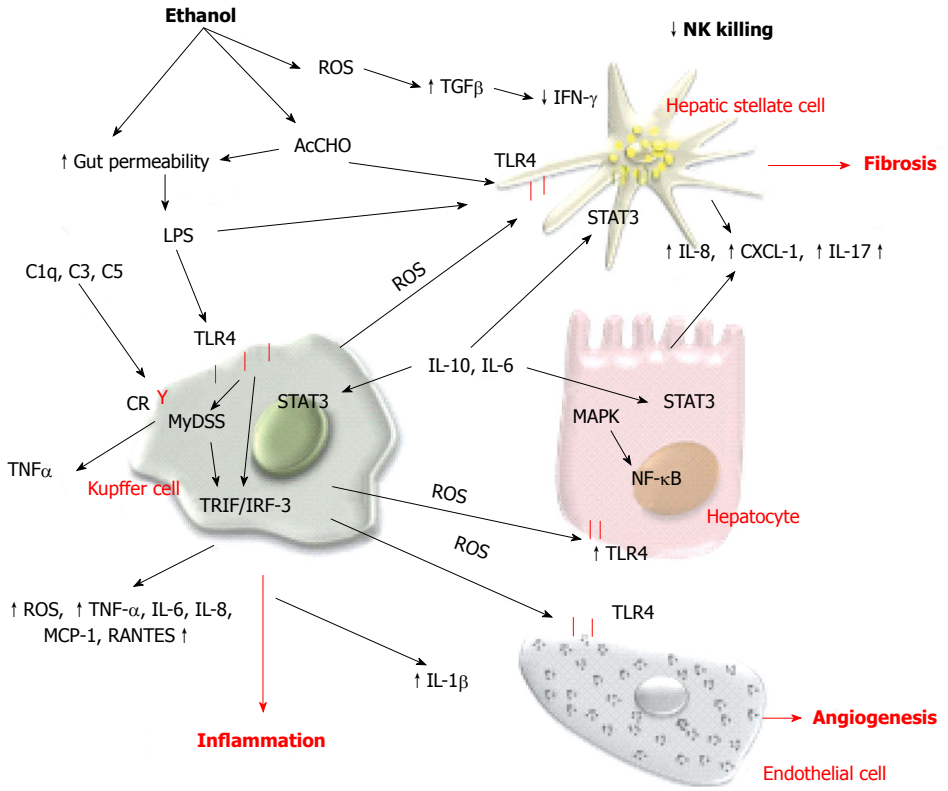

Figure 4 Alcohol and innate immune response. Both alcohol and acetaldehyde increase the intestinal permeability and lipopolysaccharide (LPS) level in the portal circulation. LPS binds to TLR4 and induces the proinflammatory phenotype of Kupffer cells. Acetaldehyde and LPS also stimulate parenchymal and nonparenchymal cells to produce proinflammatory cytokines and chemokines. The innate immune system also releases anti-inflammatory and hepatoprotective cytokines that activate STAT3 signaling in liver cells. ROS: Reactive oxygen species; LPS: Lipopolysaccharide; TLR4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-α; IFN-γ: Interferon-γ; IL: Interleukin; NF-κB: Nuclear factor-κB.

some subunit LMP7. The proteasome suppression can alter the processing of antigenic proteins and in turn affect the presentation of these antigens to cells of adaptive immunity[181]. Furthermore, altered antigen presentation has also been shown in dendritic cells where ethanol inhibits exogenous and allogeneic antigen presentation and affects the formation of peptide-major histocompatibility complex (MHC)-Ⅱ complexes, as well as altering co-stimulatory molecule expression on the cell surface[182]. Chronic ethanol consumption downregulates the number of $F4/80^+$ cells expressing MHC-Ⅰ and -Ⅱ. Elimination of TLR4 abolishes the effects of ethanol on the adaptive inflammatory response in macrophages, suggesting that alterations in TLR4 function might modulate interaction between innate and adaptive immune responses in ALD[183].

ALCOHOL AND HEPATOCARCINOGENESIS

Alcohol consumption is a risk factor for epithelial cancers including HCC. Although DNA-adducts with aldehydes generated from ethanol oxidation are involved in mutagenesis and carcinogenesis[184], cirrhosis is the principal risk factor for HCC. The mechanisms that contribute to development of HCC in patients with cirrhosis are complex and include telomere shortening, activation of pathways that promote tumor cell survival, proliferation, loss of cell cycle checkpoints, and activation of oncogenes[185,186].

In addition, the immunosuppressive effects of alcohol[185,186] contribute to the development of HCC in patients with ALD[187-189]. Recently, interesting data about epigenetic regulation in ALD have been published. Epigenetic alterations by alcohol include histone modifications such as changes in acetylation, phosphorylation, hypomethylation of DNA, and alterations in different miRNAs. Deregulation of miRNA biogenesis has been found in nonviral HCC subtypes, and ethanol oxidation influences the expression of miR-217, miR-155 and miR-212[190]. These modifications can be induced by oxidative stress that results in altered recruitment of transcriptional machinery and abnormal gene expression. Epigenetic mechanisms play an extensive role in the development of liver cancer, contributing to the reversion of normal liver cells into progenitor and stem cells. In the alcohol-preferring rat model, heavy alcohol ingestion amplifies age-related hepatocarcinogenesis but does not cause appreciable liver inflammation or fibrosis. In these animals, alcohol exposure activates the Hedgehog pathway and induces related procarcinogenic processes such as deregulated progenitor expansion, and epithelial-

mesenchymal transition[190,191]. *In vivo* and *in vitro* alcohol exposure induces chromosomal aberration and mitotic targets such as cyclin B, aurora kinase A, and phosphorylation of γ-tubulin[191].

THERAPEUTIC OPTIONS

Alcohol cessation is the mainstay of therapy for patients with all stages of ALD, however different drugs that target specific pathways have been proposed for ALD treatment. Oxidative stress plays a central role in the pathogenesis of ALD, and several preclinical and clinical trials with antioxidant agents have been performed. N-acetylcysteine (NAC), S-adenosyl methionine (SAMe), *Silybum marianum* (*Cardus marianum* L.), and vitamin E have been tested either in combination with glucocorticoids or as a monotherapy. NAC and SAMe have failed to demonstrate any benefit in the outcome of ALD[192,193] but may offer additional incremental benefit when combined with prednisolone[194]. *S. marianum* or milk thistle (MT) is the most well-researched plant in the treatment of liver disease and has been used to treat ALD and acute and chronic viral hepatitis. In baboons, the active principle called silymarin, administered for 3 years, retarded the development of alcohol-induced hepatic fibrosis[195]. The major mechanism of its hepatoprotective activity is the inhibition of hepatic NF-κB activation. In addition, silymarin has antifibrotic activity in rodents and inhibits the expression of pro-collagen-α1(I) and tissue inhibitor of metalloproteinase-1 *via* downregulation of TGF-β1 mRNA[196]. Silymarin acts as antioxidant; it reduces free radical production and lipid peroxidation and markedly increases the expression of superoxide dismutase in lymphocytes of patients with alcoholic cirrhosis[197,198]. Silymarin also shows anti-inflammatory and antiangiogenic effects[199]. However, clinical trials have not been encouraging. In a double-blind comparative study of 106 patients with histological alcoholic hepatitis, MT showed no positive effects on liver biopsy[200]. The Cochrane Library does not recommend the use of MT for acute or chronic alcoholic liver injury and recommends conducting new randomized controlled clinical trials[201].

Studies with other antioxidants such as vitamin E and propylthiouracil have likewise been disappointing[194,202-204], while animal data on Isoorientin[205] and Notoginseng[206] are encouraging but further studies are needed.

Deregulation of PPAR transcriptional activity during alcohol consumption suggests a possible role of PPAR agonists for ALD treatment. In alcohol-treated mice, the PPARγ agonists, rosiglitazone and pioglitazone, increase circulating levels of adiponectin and expression of its receptors in the liver that is associated with SIRT1-AMPK signaling activation. This pathway correlates with the enhanced expression of fatty acid oxidation enzymes and reduction of alcohol-induced steatosis[207-213]. In addition, PPARγ agonists have anti-inflammatory effects that reduce cytokine expression such as TNF-α, IL-6 and MCP-1 in alcohol-fed mice[207].

The altered intestinal microflora during chronic alcohol consumption has recently been focused as a therapeutic target in ALD. Chronic ethanol feeding causes a decline in the abundance of Bacteriodetes and Firmicutes phyla, with a proportional increase in the Gram-negative Proteobacteria. Oral administration of *Lactobacillus rhamnosus* GG attenuates the established alcohol-induced hepatic steatosis and liver injury in mouse models of ALD[208]. Probiotics create an anti-inflammatory milieu, decrease production of proinflammatory bacterial products, and improve barrier integrity leading to a decrease of endotoxin release. These protective effects are correlated with the prevention of alcohol-induced oxidative stress, suppression of CYP2E1 expression, inactivation of TLR4, and inhibition of p38 MAPK phosphorylation, which leads to a significant decrease in NF-κB activation and TNF-α production[209]. Results from a placebo-controlled trial have recently shown that the nonabsorbable antibiotic rifaximin modifies the gut microbiota, and protects alcoholic patients from hepatic encephalopathy[210,211]. Similar results have been seen with TLR4 antagonists, which have been recently studied as therapeutic agents for chronic liver diseases, including ALD[212].

Anti-inflammatory therapy remains the most attractive approach for ALD. Glucocorticoid therapy was first demonstrated to be beneficial in patients with severe AH in 1978[213]. Steroids ameliorate liver inflammation and systemic inflammatory responses, however, this treatment inhibits liver regeneration and does not promote liver repair in patients with ALD, which may contribute to the lack of long-term survival benefit in patients with severe alcoholic hepatitis. On the contrary, Anti-TNF-α therapy has demonstrated positive effects in animal models of alcoholic liver injury. Patients with severe AH have high concentrations of TNF-α[214] and the serum levels of this cytokine predict short-[215] and long-term survival[216]. In rats with experimental alcoholic steatohepatitis, infliximab, an anti-TNF-α mouse/human chimeric antibody, acts as an effective hepatoprotective and anti-inflammatory agent, and significantly improves hepatic inflammation[217]. However, a randomized double-blind placebo-controlled trial in patients with AH, using etanercept, a p75-soluble TNF receptor, failed because of the high mortality rate[218]. In severe AH, single-dose infliximab is associated with improved survival, but infection remains the main complication and large randomized controlled trials are needed before this anti-TNF-α agent can be recommended for AH[219]. A moderate effect on TNF-α levels was also demonstrated using pentoxifylline, a nonselective phosphodiesterase inhibitor[220-222] that exerts antifibrogenic action *via* downregulation of TGF-β1 expression[223].

Interesting data about the protective role of IL-22 in ALD have recently been published. IL-22 is a member of the IL-10-like cytokine family that is produced by T-helper 17 and NK cells. IL-22 has an important role in controlling bacterial infection, homeostasis, and tissue

repair[224,225]. The biological effects of IL-22 are mediated through binding to IL-22 receptors and consequent activation of the STAT3 signaling pathway[224]. IL-22 protects against liver injury[226-231], reduces fat accumulation and collagen deposition[231-234] and promotes liver regeneration[235,236] in rodent models of ALD. The antifibrotic properties of IL-22 depend on the significant increase of STAT-mediated HSC senescence, as demonstrated by the increase of β-galactosidase-positive HSCs in IL-22-treated animals[237]. Data showing elevated IL-22 expression in ALD patients suggest that IL-22 administration might be an ideal therapy for alcoholic liver injury[238].

Inhibition of hepatocyte apoptosis was recently suggested as an alternative and attractive approach to reduce liver inflammation during alcohol consumption. The pancaspase inhibitor emricasan was found to suppress hepatocyte apoptosis, inhibit proinflammatory caspases, and prevent fibrogenesis in murine models of ethanol-induced liver injury[239].

Many other chemokines (*e.g.*, CXCL5, CXCL6 and CXCL4) and cytokines (*e.g.*, IL-1, IL-8 and OPN) are markedly upregulated in AH and are implicated in the hepatic neutrophil infiltration[162-164]. Reagents that target CXC chemokines are currently under investigation in different stages of ALD.

CONCLUSION

Chronic alcohol consumption is a major cause of advanced liver disease worldwide. In this review, we have highlighted the role of alcohol abuse in liver disease by examining ethanol metabolism. Both acetaldehyde and ROS act directly on the transcriptional network that regulates lipid metabolism and fibrogenic response during liver injury. These toxic agents alter the intestinal permeability and consequently increase LPS, which leads to the activation of innate and adaptive immunity. LPS activation of TRL4 stimulates Kupffer cells to release ROS and cytokines that attract neutrophils, inhibits NK function, and alter allogenic antigen presentation. In addition, acetaldehyde and ROS promote a chronic inflammatory state that has a direct role in the development of HCC. Furthermore, lipid peroxidation products and the formation of protein and DNA adducts interfere with methylation, synthesis and repair of DNA and promote mutagenesis. The specific pathways involved in ethanol-induced liver damage select new therapeutic agents such as thiazolidinediones, anti-TNF-α molecules and IL-22 that have shown promising effects in basic and translational research studies. Future efforts should be directed to test the new therapeutic approaches in controlled clinical trials in patients with moderate and severe ALD.

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REVIEW

Procalcitonin and intestinal ischemia: A review of the literature

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Abstract

Intestinal ischemia is common after emergency gastrointestinal or cardiovascular surgery. At present, there are no diagnostic tools for the early diagnosis of intestinal ischemia. In the last decade, procalcitonin (PCT) has been suggested as a marker of this condition. Here, we review the use of PCT as a diagnostic tool for intestinal ischemia. Two reviewers independently searched the PubMed and EMBASE databases for articles on intestinal ischemia and PCT. They then considered (1) the criteria applicable to preclinical and clinical data; and (2) PCT's predictive value in the diagnosis of intestinal ischemia. Article quality was rated according to the STAndards for Reporting of Diagnostic accuracy. Between 1993 and 2014, seven studies (including two preclinical studies and five clinical studies) dealt with the use of PCT to diagnose intestinal ischemia. Procalcitonin's sensitivity, specificity, positive predictive value and negative predictive value ranged between 72% and 100%; 68% and 91%; 27% and 90% and 81% and 100%, respectively. The area under the receiver operating characteristic curve ranged from 0.77 to 0.92. In view of the preclinical and clinical data, we consider that PCT can be used in daily practice as a tool for diagnosing intestinal ischemia.

Key words: Procalcitonin; Intestinal; Ischemia; Diagnosis; Review

Core tip: The serum procalcitonin level is clinically relevant for the diagnosis of intestinal ischemia. In the diagnosis of intestinal ischemia, procalcitonin's sensitivity is greater than 70% and its negative predictive value is greater than 80%.

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INTRODUCTION

Intestinal ischemia is a common complication of intestinal diseases (*e.g.*, small bowel obstruction and ischemic colitis) that can occur during disease progression (either

spontaneously or after abdominal or cardiovascular surgery)[1,2]. The condition has been defined as impairment of the intestinal blood supply from the celiac axis, the superior mesenteric artery and the inferior mesenteric artery; this results in tissue injury and a low-flow state with poor intestinal arterial perfusion[3,4]. In the United States, intestinal ischemia accounts for 0.1% of all hospital admissions. The incidence of this condition has increased over the last few decades (from 1 in 1000 to 1 in 200 hospitalizations for abdominal pain). In most cases, intestinal ischemia requires emergency treatment to avoid tissue necrosis, infectious outcomes, septic shock or potentially lethal multiple organ failure. This is notably the case in ischemic colitis (the most frequent gastrointestinal vascular disease, the incidence of which ranges from 4.5 to 44 cases per 100000 persons per year)[5].

Management of intestinal ischemia is difficult because the clinical symptoms associated with this condition (mild but sudden pain, diarrhoea, low gastrointestinal bleeding, abdominal distension with vomiting, fever, tachycardia, and tachypnoea) are not specific enough to rule out a number of other differential diagnoses[6]. In addition to the clinical symptoms, tissue damage be assessed using computed tomography angiography, Doppler ultrasound and endoscopy[7]; however, the lack of sensitivity and specificity associated with these examinations may mean that the diagnosis is only confirmed during the surgical procedure (*i.e.*, too late)[8].

With a view to improving the preoperative diagnosis, some researchers have suggested measuring several biomarkers: L and D-lactate[9-11], leukocytes[11,12], α glutathione S-transferase (αGST)[12-14], diamine oxidase[15], trehalase[16], alcohol dehydrogenase[17]), intestinal fatty acid binding protein[18-20], and D-dimer[21]. Whilst most of these markers prove to be accurate in preclinical studies, their use in clinical practice has been limited by several shortcomings (lack of sensitivity and specificity; poor assay reproducibility and the presence of species-specific metabolites). Hence, a clinical consensus on the use of these substances has not been reached.

Over the last decade, procalcitonin (PCT) has been suggested as a novel biomarker of the tissue damages associated with intestinal ischemia[22,23]. Procalcitonin is a 12.6 kDa, 116-amino-acid (AA) precursor of calcitonin that was first described in 1993 by Assicot *et al*[24] as a marker of infection. It has three domains: a 57 AA N-terminal domain, the 32 AA calcitonin fragment (involved in the regulation of calcium and phosphorus metabolism) and the 21 AA katakalcin fragment (measured in PCT assays)[24]. Procalcitonin is a member of the calcitonin gene-related peptide family and is encoded by the *CALC-1* gene located on the chromosome 11 (11p)[24-26].

In healthy subjects, the "hormokine" PCT is released from the C cells of the thyroid[27]. In a disease context, PCT production can be stimulated by trauma[28], bacterial endotoxins, pro-inflammatory cytokines [tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6)] or cardiogenic shock[24]. It is thought that this PCT is released by the liver parenchyma[29]. In most laboratories, PCT levels are measured with the Kryptor® TRACE assay. The normal level (from the third postnatal day onwards) is below 0.05 ng/mL. After surgery, the PCT level can rise to as much as 1 ng/mL (in cases of minor or aseptic surgery) or even 2 ng/mL (in cardiac surgery)[30,31].

The half-life-time of PCT is between 18 and 24 h, in patients with kidney failure, between 24 and 30 h (with a peak at 24 h)[31]. As shown by Meisner *et al*[32], the kinetics of serum PCT are not influenced by age, gender or renal function (because only a proportion of PCT is excreted by the kidneys).

Here, we review the literature data on the use of PCT in the diagnosis of intestinal ischemia.

RESEARCH

Search strategy and selection criteria

Two reviewers independently searched the PubMed and EMBASE databases for articles related to intestinal ischemia and PCT and published between 1993 and 2014. The search terms were "intestinal ischemia", "gut ischemia", "mesenteric ischemia" and "procalcitonin". Only full, original articles written in English were selected. For each selected article, the list of references was checked for studies not listed in the PubMed and EMBASE databases or not found in the search.

Data extraction and analysis

The two reviewers extracted the following data from each selected study: first author, date, type of study (preclinical or clinical), the number of included patients and the diseases assessed. The data were separated into two categories: those related to PCT's characteristics (structure, pharmacokinetics and pharmacodynamics), and those related to the detection of intestinal ischemia by measuring PCT (thresholds and predictive values). All extracted data were recorded in a table.

Assessment of the quality of selected publications

The methodological quality of the diagnostic studies was independently evaluated by the two reviewers according to the STAndards for Reporting of Diagnostic accuracy (STARD) criteria[33]. The studies were graded based on items relevant for this review. Studies were divided into groups as a function of the calculated STARD score; a score of 8 or 9 indicates good quality; a score of 6 or 7 indicates fair quality and a score of 5 or less indicates poor quality.

Any disagreements between the two reviewers over data quality were resolved by consensus.

THE DIAGNOSTIC VALUE OF PROCALCITONIN

According to our search results, a total of 32 studies related to PCT and ischemia were published between 1993

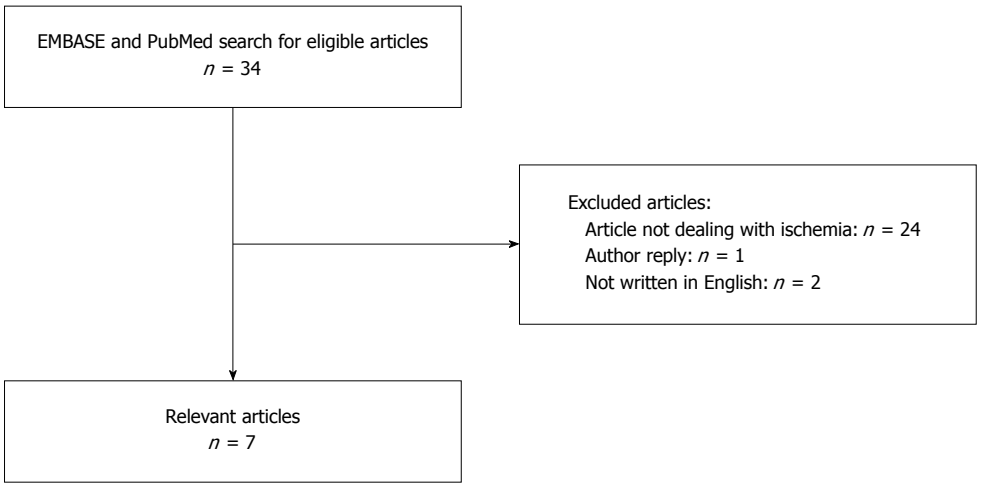

Figure 1 Review'S flow scheme.

Table 1 Preclinical data on procalcitonin for the diagnosis of intestinal ischemia

Ref.	Model	Number of animals	Model of ischemia	Range of procalcitonin values (ng/mL)	Assessment methods
Ayten *et al*[34]	New Zealand rabbits	30	Ligature of a 10-cm distal ileum segment and (in some cases) the mesenteric artery	0.225-0.514	Immunoluminometry
Karabulut *et al*[35]	New Zealand rabbits	21	Ligature of the superior mesenteric artery	0.11-0.98	ELISA (kit E0689)

Table 2 Clinical data on procalcitonin for the diagnosis of intestinal ischemia

Ref.	Condition	Number of patients	Range of procalcitonin values (ng/mL)	Assay method
Nagata *et al*[36]	Open aortic surgery	93	< 0.5 - > 10	Immunochromatographic test
Markogiannakis *et al*[22]	Small and large bowel obstruction	242	4.89-14.35	LUMItest
Cosse *et al*[23]	Small bowel obstruction	166	0.29-2.03	Kryptor TRACE
Cosse *et al*[37]	Small bowel obstruction	59	0.06-8.1	Kryptor TRACE
Cosse *et al*[38]	Ischemic disease (ischemic colitis and mesenteric infarction)	99	0.217-621.2	Kryptor TRACE

and 2014 (Figure 1). Only seven of these studies were relevant. All seven were of fair quality (*i.e.*, with a STARD score of between 6 and 7).

Preclinical data

Only two groups reported on the use of PCT as a diagnostic tool for bowel ischemia in an animal model (Table 1). In 2005, Ayten *et al*[34] studied 30 New Zealand rabbits in which intestinal ischemia was induced by ligature of a 10-cm distal ileum segment and (in some cases) ligature of the mesenteric artery. The researchers used an immunoluminometric method to measure serum PCT values during ischemia, which ranged from 0.22 to 0.51 ng per mL. In 2011, Karabulut *et al*[35] reported on a study in which intestinal ischemia was induced in 21 New Zealand rabbits by ligature of the superior mesenteric artery. The serum PCT level was measured with an ELISA (the E0689 kit) and ranged from 0.11 to 0.98 ng per mL.

Clinical data

The use of PCT in clinical practice has been investigated by three groups in a total of 659 patients (Table 2). In 2007, Nagata *et al*[36] evaluated the value of PCT for diagnosing colonic ischemia in a cohort of 93 patients undergoing open aortic surgery. The PCT levels were assayed using an immunochromatographic method and ranged from below 0.5 ng/mL in patients without ischemia to > 10 ng/mL in patients with ischemia. In 2011, Markogiannakis *et al*[22] suggested that PCT could be used as a diagnostic tool for ischemia and necrosis on the basis of their study of 242 patients treated for small or large bowel obstruction due to various aetiologies. According to the LUMItest, the PCT levels in patients with ischemia or necrosis ranged from 4.89 ng/mL to 14.35 ng/mL. In 2013, our group investigated the value of measuring PCT in the management of small bowel obstruction in a cohort of 166 patients from a randomized

Table 3 Diagnostic utility of procalcitonin for the diagnosis of intestinal ischemia

Ref.	Outcome	Threshold (ng/mL)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Area under the curve
Nagata *et al*[36]	Colonic ischemia	2	100	83.9	27	100	NR
Markogiannakis *et al*[22]	Bowel ischemia	0.25	72	73	60	83	0.77
Markogiannakis *et al*[22]	Bowel necrosis	0.25	83	78	52	95	0.87
Cosse *et al*[23]	Small bowel ischemia	0.57	83.3	91.3	83.3	91.3	0.91
Cosse *et al*[37]	Small bowel ischemia	0.53	80	84.8	40	90.7	0.86
Cosse *et al*[38]	Bowel necrosis	2.47	94.6	68	89.8	80.9	0.92

NR: Not been reached.

clinical trial[23]. We reported that when measured with the Kryptor TRACE method, the PCT values ranged from 0.29 ng/mL to 2.03 ng/mL in patients with ischemia. Moreover, we validated these data in a second, distinct cohort of 59 patients treated for small bowel obstruction by showing that PCT values (measured with the same assay) ranged from 0.06 ng/mL to 8.1 ng/mL in individuals with intraoperatively confirmed ischemia[37]. Our group also evaluated the value of PCT for characterizing tissue damage in cases of intestinal ischemic diseases (including ischemic colitis and mesenteric infarction)[38]. In a cohort of 99 patients, we found that PCT values ranged from 0.217 to 621.2 ng/mL when necrosis was described in the pathologist's report.

Diagnostic value

A variety of PCT upper threshold values have been reported for the diagnosis of ischemia and necrosis (Table 3). Even though the thresholds differed from one study to another, the predictive characteristics were similar. For example, the sensitivity ranged from 72% to 100%. The lowest values (72%-83%) were found for the lowest thresholds (0.25-0.57 ng/mL) in small bowel ischemia, whereas the highest values (95% and 100%) are found for high thresholds (> 2 ng/mL, *i.e.*, four times the normal upper limit) in colonic ischemia and necrosis. Furthermore, the clinical data were also associated with high negative predictive values (NPVs). With relatively low thresholds, the NPVs ranged from 81% to 100% for the diagnosis of ischemia and necrosis. The fact that the area under the curve (AUC), was greater than 0.75 emphasizes the clinical relevance of PCT as a diagnostic tool under these conditions.

NEED FOR NEW BIOMARKERS

Here, we reviewed the preclinical and clinical data on the use of PCT as a diagnostic tool for intestinal ischemia. All these data argue in favour of PCT as a new biomarker for this condition.

The diagnostic value of PCT in ischemia in general (and intestinal ischemia in particular) was suggested some time ago on the basis of two studies in rabbits. This concept has since matured in the scientific community. Indeed, acute intestinal ischemia is a life-threatening condition that requires emergency treatment and so must be diagnosed as soon as possible. To this end, clinicians can use biomarkers. The best-defined biomarkers are the D-lactate level and the leukocyte count. Murray *et al*[9] (1994) and Poeze *et al*[10] (1998) showed that D-lactate has a sensitivity of 80% for diagnosing ischemia, whereas Evenett *et al*[11] (2009) and Delaney *et al*[12] (1999) reported that the leukocyte count is more relevant. However, these "old" markers are used less and less because their accuracy is subject to debate. Although other molecules (such as αGST and D-dimer) have been suggested as alternatives to D-lactate and leukocytes, their value has yet to be established; this has prompted a search for other candidates[13-14,21].

As shown in clinical studies, PCT is a promising biomarker for the diagnosis of intestinal ischemia. Indeed, the groups working on PCT have reported that it has high diagnostic value. With low thresholds (0.25-0.5 ng/mL), the serum PCT value provides the clinician with information on the presence of reversible ischemic injuries and can identify patients requiring emergency treatment or those requiring close monitoring. The good agreement between the various groups' findings and the high reported AUC values (even though the assay technique varied from one study to another) underline the reproducibility of PCT assays and means that the clinician can envisage use of the latter in clinical practice.

ROLE OF PROCALCITONIN IN THE PHYSIOPATHOLOGY OF ISCHEMIA

Procalcitonin's value in the diagnosis of intestinal ischemia may perhaps be explained by the physiopathology of this condition. Ischemia is defined as a decrease in blood flow through the vessels. An inflammatory reaction then triggers the release of reactive oxygen species, which in turn promote the secretion of TNF-α and IL6. The resulting oxidative stress affects the intestinal mucosa and enterocytes, thus reducing the permeability of the intestinal wall. The indigenous bacteria in the gastrointestinal tract (*Escherichia coli*, *Lactobacillus* species, *Klebsiella, Bacteroides* species, *etc.*,) proliferate and generate bacterial endotoxins that ultimately promote the release of PCT

into the blood stream[39].

WEAKNESSES AND STRENGTHS OF PROCALCITONIN AS A BIOMARKER

Given that PCT was first studied as a marker of infection, its diagnostic value in intestinal ischemia might conceivably be limited by the presence of a bacterial infection. Indeed, several groups have reported that PCT is a diagnostic marker for (1) the sepsis[40-42] that can occur during ischemia; and (2) the necrosis related to multiple organ failure. When faced with suspected ischemic injuries and a high PCT value, the physician should request a microbiological analysis to rule out infection. Indeed, ischemic phenomena are closely related to infectious phenomena because PCT secretion depends respectively on inflammation and on bacterial translocation leading to infection. Chronic kidney disease might also conceivably influence PCT values, although Meisner *et al*[32] have reported that this is not the case. Moreover, PCT levels may be elevated by some non-ischemic phenomena (cardiac arrest, drug reaction with eosinophilia and systemic symptoms syndrome, heat wave, *etc.*) and decreased by others (previous effective antibiotic therapy, tuberculosis, *etc.*). To counterbalance these shortcomings of PCT, new biomarkers (such as copeptin and proadrenomedulin) should be investigated in ischemic conditions associated with PCT release. Moreover, the discordance between preclinical models and differences in the aetiologies of ischemia in clinical studies may be confounding factors in the assessment of PCT's diagnostic value.

Nevertheless, the findings of preclinical studies are generally in agreement (*i.e.* with the same trends in similar animal models). Furthermore, the clinical data support the use of PCT as a relevant tool for the diagnosis of ischemia (regardless of the aetiology) with thresholds of 1-2 ng/mL).

CONCLUSION

In view of the available preclinical and clinical data, we consider that PCT can be used in daily practice as a tool for diagnosing intestinal ischemia.

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REVIEW

Helicobacter heilmannii sensu lato: An overview of the infection in humans

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Abstract

Helicobacter heilmannii sensu lato (*H. heilmannii* s.l.) is a group of gastric non-*Helicobacter pylori Helicobacter* species that are morphologically indistinguishable from each other. *H. heilmannii* s.l. infect the stomach of several animals and may have zoonotic potential. Although the prevalence of these infections in humans is low, they are associated with gastric pathology, including mucosa-associated lymphoid tissue lymphoma, making them a significant health issue. Here, the taxonomy, epidemiology, microbiology, diagnosis, and treatment of these infections will be reviewed. The gastric pathology associated with *H. heilmannii* s.l. infections in humans will also be addressed. Finally, the features of the complete bacterial genomes available and studies on species-specific pathogenesis will be reviewed. The understanding of the mechanisms that underlie gastric disease development mediated by the different bacterial species that constitute *H. heilmannii* s.l. is essential for developing strategies for prevention and treatment of these infections.

Key words: *Helicobacter heilmannii sensu lato*; Gastric non-*Helicobacter pylori; Helicobacter* species; Pathogenesis; Diagnosis; Treatment; Genomes

Core tip: *Helicobacter heilmannii sensu lato* is a group of non-*Helicobacter pylori Helicobacter* species that infect the stomach of animals and humans. In the human stomach, these infections are associated with several pathologies, but it is currently unknown whether certain species are more often associated with a certain disease outcome than others. The access to bacterial genomes together with the availability of increasing numbers of *in vitro* isolates will allow significant advances in the understanding of species-specific bacteria-host interactions in disease pathogenesis and will be essential for future development of strategies to prevent and treat these infections.

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INTRODUCTION

The first descriptions of spiral bacteria colonizing the stomach of animals date from the end of the 19th century[1], and reports of such microorganisms in the human stomach date from the beginning of the 20th century[2]. Also, by that time, the presence of urease activity in the stomach was reported[3], but no associations were made between this observation and the presence of microorganisms in the stomach. The occurrence of spirochetes in stomachs from autopsied individuals and in fresh gastric surgical specimens was reported later[3,4]. None of these findings received much attention, as the stom-

Table 1 Natural hosts and characteristics of *Helicobacter heilmannii* s.l. species that infect humans

Species	Natural host	Length/width (μm)	Number and distribution of flagella	Periplasmatic fibrils	*In vitro* isolation from humans	Ref.
H. heilmannii s.l.						
H. bizzozeronii	Cat, dog, fox ,lynx	5-10/0.3	10-20, bipolar	No	Yes	[17,42,81,106]
H. felis	Cat, dog, rabbit, cheetah	5-7.5/0.4	10-17, bipolar	Yes	Yes	[17,45,68,80]
H. heilmannii s.s.	Cat, dog, fox, lynx, non-human primates	3.0-6.5/0.6-0.7	4-10, bipolar	No	No	[15,17,82,106]
H. salomonis	Cat, dog, rabbit	5-7/0.8-1.2	10-23, bipolar	No	No	[17,43,106]
H. suis	Pig, non-human primates	2.3-6.7/0.9-1.2	4-10, bipolar	No	No	[17,44,83]
H. pylori		2.5-5.0/0.5-1.0	4-8, unipolar	No	Yes	[6]

ach was considered sterile and a hostile environment for bacteria. This view started to change only in 1982, when Marshall and Warren[5] successfully cultured *Helicobacter pylori* (*H. pylori*), a spiral-shaped, Gram-negative bacterium from a gastric biopsy specimen. Further studies have since shown that *H. pylori* is the most common chronic infection in humans, and established this species as the main etiologic factor in peptic ulcer disease, gastric carcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma[6]. Since the discovery of *H. pylori*, many new *Helicobacter* species that infect human or animal hosts have been described, and the *Helicobacter* genus now includes more than 30 formally named species[7]. Here, we will review the gastric non-*H. pylori Helicobacter* species generally referred to as "*Helicobacter heilmannii*", focusing on those that infect humans and on their impact in human disease.

TAXONOMY

A spiral-shaped bacterium colonizing the human gastric mucosa that was different from *H. pylori* was reported for the first time by Dent *et al*[8] in 1987. Two years later, the same authors described this bacterium and proposed a new genus and species-*Gastrospirilum hominis*[9]. Later 16S rRNA gene sequencing analysis led to its reclassification within the *Helicobacter* genus[10]. It was then provisionally renamed as *Helicobacter heilmannii* (*H. heilmannii*), in acknowledgement of Konrad Heilmann, the German pathologist who first studied the pathologic features of this infection in the human stomach[11]. Further 16S rRNA analysis of an increasing number of samples led to the sub-classification of *H. heilmannii* into type 1 and type 2[10,12]. It was shown that *H. heilmannii* type 1 represented a single species, *H. suis*, that colonizes the stomachs of pigs[13], whereas *H. heilmannii* type 2 represented a group of species that colonize the stomachs of cats and dogs and includes *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis*, and *H. heilmannii* sp. nov.[14]. The latter had been provisionally named "Candidatus *H. heilmannii*" in 2004, based on urease gene sequence analysis and because it could not be cultured *in vitro* at that time[14]. In fact, only very recently and after successful *in vitro* isolation, *H. heilmannii* was formally recognized as a valid species name[15].

To avoid further confusion in nomenclature, in 2011 the introduction of the terms *Helicobacter heilmanni sensu lato* (*H. heilmanni* s.l.) was proposed to refer to the non-*H. pylori Helicobacter* species detected in the stomachs of humans or animals if only histopathological, electron microscopy, or crude taxonomic data are available; and *Helicobacter heilmannii sensu stricto* (*H. heilmanni* s.s.) or the other species names if definite identification at the species level is achieved[16].

EPIDEMIOLOGY AND TRANSMISSION

As many as 11 different *H. heilmannii* s.l. have been described colonizing the stomach of domesticated and wild animals[17], 5 of which have been found in the human stomach, namely *H. suis*, *H. felis*, *H. bizzozeronii*, *H. salomonis*, and *H. heilmannii* s.s. (Table 1).

In Western countries as well as in Japan, the prevalence of *H. heilmannii* s.l. in human gastric biopsies is generally lower than 1%, both in adults and in children[18-23]. Reports from China and Thailand indicate that the prevalence of the infection can reach 2% and 6%, respectively[24,25].

Because of the nomenclature problems and due to the difficulty of cultivating these bacteria to allow species differentiation, there are few studies addressing the prevalence of each species individually. In gastric biopsy samples with histological evidence of *H. heilmannii* s.l., polymerase chain reaction (PCR)-based techniques showed that *H. suis* is the most prevalent *H. heilmannii* s.l. species infecting the human stomach, with prevalence ranging from 14% to 37%[26,27]. *H. salomonis* was present in 21%, *H. felis* in 15%, *H. heilmannii* s.s. in 8%, and *H. bizzozzeronni* in 4% of cases[27]. Infection with two or more *H. heilmannii* s.l. species or *H. pylori* and *H. heilmannii* s.l. can be present in the same gastric biopsy[28,29].

While *H. pylori* is known to colonize mainly humans and few non-human primates, *H. heilmanni* s.l. species have other non-human and probably more important hosts, such as cats, dogs, and pigs[17]. In addition to being present in the stomach, *H. heilmannii* s.l. species have also been reported in the oral cavity of domestic dogs and cats and, recently, *H. bizzozeronii* and *H. salomonis* were detected in canine saliva[30-32]. Several reports have suggested the transmission of *H. heilmanni* s.l. from pets to their owners by direct contact[33-36]. A higher prevalence of *H. heilmanni* s.l. infection among people that live in rural areas and of those who often have contact with dogs, cats, cattle, or pigs has been described[37-39]. It has

further been suggested that *H. suis* might be transmitted to humans by consumption of contaminated raw pork meat, where the bacterium can viably persist for up to 48 h[40]. Given this evidence, it has been hypothesized that *H. heilmannii* s.l. infection is a zoonosis.

MICROBIOLOGY

All *H. heilmannii* s.l. species are Gram-negative, microaerophilic, and catalase- and urease-positive[17]. The first descriptions of *H. heilmannii* s.l. used the term "corkscrew-like" bacteria because of their morphology[41]. As a group, these bacteria are microscopically very similar, with spiral and coiled shape, with 4-6 helical turns and 2.3-10 μm in length (Table 1)[11,15,42-45]. *H. heilmannii* s.l. have varying number of flagella, which have bipolar distribution, and fix themselves to a blunt undulated part of the cell wall[11]. *H. felis* is the only *H. heilmannii* s.l. species that has periplasmatic fibrils[15,42-45]. *H. suis* has several contrasting features when compared with other *H. heilmannii* s.l. species, since it may be shorter in length and have fewer flagella[15,42-45].

H. HEILMANNII S.L.-ASSOCIATED DISEASES IN HUMANS

The relationships between *H. heimannii* s.l. and disease in humans are mostly based on publications that have only identified the agent as gastric non-*H. pylori Helicobacters*.

The initial description by Dent *et al*[8] of *H. heimannii* s.l. in the gastric mucosa of 3 patients with gastritis was followed by several other publications, mostly case reports[46-49]. Since then, *H. heilmannii* s.l. infection has been reported in cases of peptic ulcer disease[19,24,39,50], gastric carcinoma[50-52], and gastric MALT lymphoma[21,50,53]. Infected patients may be asymptomatic or present dyspeptic symptoms, such as chronic epigastric pain, nausea, vomiting, heartburn, dysphagia, and post-prandial discomfort[11,19,50,54].

Heilmann and Borchard were the first to report the histopathological and ultrastructural features of a large series of 39 cases with *H. heilmannii* s.l. infection[11]. Bacteria were more frequently found colonizing the antrum although 20% of the cases presented microorganisms also in the fundus. *H. heilmannii* s.l. were found as single microorganisms or in small groups, located underneath the mucous layer, above the surface cells, and deep in the lumen of the foveolae. In ultrastructural analyses, the close contact of some bacteria with the membrane of surface mucous cells, in association with degenerative changes of the cell membrane and partial destruction of the microvilli was reported[11]. The presence of *H. heilmannii* s.l. inside mucous and parietal cells and inside parietal cell canaliculi in the corpus mucosa was also observed[11,50]. *H. heilmannii* s.l.-infected cases mostly presented mild active chronic gastritis in the antrum and mild inactive gastritis in the fundus.

Some years later, Stolte *et al*[50] undertook a study to compare the parameters of gastritis between 202 German patients infected with *H. heilmannii* s.l. and 202 matched control patients infected with *H. pylori*. In agreement with the previous findings[11], they observed that *H. heilmannii* s.l. colonization occurred predominantly in the antrum and mainly focally[50]. They also observed that the grading of the parameters of gastritis, such as the density of lymphocytic and neutrophilic infiltration, the replacement of foveolae by regenerative epithelium, and mucus depletion were significantly milder in *H. heilmannii* s.l. infection than in *H. pylori* infection. Additionally, the presence of lymphoid follicles and intestinal metaplasia were less common in *H. heilmannii* s.l. gastritis[50]. Similar findings were reported in studies comparing the histopathological changes in the gastric mucosa between *H. heilmannii* s.l. and *H. pylori* infections in patients from other geographic areas, including Thailand, Japan, and Korea[21,24,55].

Interestingly, Stolte *et al*[50] observed a relatively high prevalence of gastric MALT lymphoma in *H. heilmannii* s.l. gastritis (3.5%), which prompted them to investigate their material from a 10-year period[56]. They observed 8 MALT lymphomas among patients with *H. heilmannii* s.l. gastritis (1.5%) in comparison with 1745 MALT lymphomas among 263680 patients with *H. pylori* gastritis (0.7%), suggesting that patients infected with *H. heilmannii* s.l. develop MALT lymphoma more frequently than those with *H. pylori*[56].

Although in the previous studies there was not a clear identification of the bacteria at a species level, in experimentally infected animal models the administration of *H. heilmannii s.s.*-positive gastric biopsy homogenates to BALB/c and to C57BL/6 mice induced gastric MALT lymphoma[57-59]. Furthermore, infection with pure bacterial isolates of *H. felis*[60-62] and of *H. suis*[63] were shown to induce gastric MALT lymphoma-like lesions in the BALB/c and in the Mongolian gerbil models, respectively.

The contribution of *H. heilmannii* s.l. to the pathogenesis of the aforementioned diseases is highlighted in reports in which eradication treatment of the bacteria is followed by symptomatic relief[11,36] and complete regression of the infection-associated lesions[11,64], including low-grade gastric MALT lymphoma[21,53,55].

DIAGNOSIS

The diagnosis of *H. heilmannii* s.l. infection poses a complex challenge in comparison to the well-established tests for *H. pylori*. The diagnosis of *H. pylori* can be achieved by non-invasive tests, which are based on detection of antibodies, bacterial antigens, or urease activity in samples such as blood, breath or stools; and invasive tests, which involve an endoscopy with collection of gastric biopsy specimens for histology, culture, urease test, or molecular methods[6].

The diagnosis of *H. heilmannii* s.l. has been based mainly on histological detection, and for this, silver staining-based techniques, such as the Steiner and the Whartin-Starry stains are preferable to hematoxylin and eosin[41].

There are currently no specific antibodies available for immunohistochemical detection of *H. heilmannii* s.l.[65]. Importantly, and although there may be morphological differences in size, number of spirals, and tightness of coils among *H. heilmannii* s.l species, and between these and *H. pylori*, these criteria are not accurate for species identification, as different species may be morphologically very similar, and variation in morphology within a single species may also occur[9,11,17,41].

The use of rapid urease tests allowing the detection of urease activity directly in the gastric biopsy specimens may not be sensitive enough[66], as the colonization density of *H. heilmannii* s.l. is lower than that of *H. pylori*, and also will not be helpful for species identification.

The use of *in vitro* culture as a diagnostic test is also not feasible due to the very fastidious nature of these bacteria. So far, very few laboratories have succeeded in the isolation of *H. heilmannii* s.l. from the gastric mucosa of cats, dogs, or pigs[15,44,45,67] and only *H. bizzozeronii* and *H. felis* have been isolated from the human gastric mucosa[35,68-70].

Currently, the most accurate method available for conclusive species identification is the use of PCR, followed by sequencing of specific target genes. These include the urease A and B (*ureA*, *ureB*) genes, the heat shock protein 60 (*hsp60*) gene, and the gyrase subunit B (*gyrB*) gene[44,71-74]. Sequencing of the 16S rRNA gene and of the 23S rRNA gene allows distinction of *H. suis* from the rest of the *H. heilmannii* s.l. species[13,44].

TREATMENT

H. heilmannii s.l. eradication treatment is indicated in patients that present with severe pathology and clinical symptomatology associated with the infection[17]. No randomized trials have been performed to evaluate the most suitable treatment for *H. heilmannii* s.l. infection. The treatment strategies used are identical to the triple therapy regimen for *H. pylori* eradication, which include a proton pump inhibitor and clarithromycin combined with amoxicillin or metronidazole for 2 wk[64,75,76]. An *in vitro* antimicrobial susceptibility study of *H. bizzozeronii*, *H. felis*, and *H. salomonis* isolates obtained from cats and dogs showed that they were sensitive to ampicillin, clarithromycin, and tetracycline, among other pharmacological agents[77]. However, acquired resistance to metronidazole was observed for some *H. bizzozeronii* and *H. felis* isolates[77]. More recently, it was confirmed that *H. bizzozeronii* had a rapid *in vitro* acquisition of resistance to metronidazole, which should be taken into account when treating this species[78]. In a mouse model of infection used for evaluating the antibiotic susceptibility of 2 different *H. suis* isolates to amoxicillin and omeprazole, a difference in susceptibility between the bacterial isolates was observed[79].

COMPLETE GENOMES OF H. SUIS, H. FELIS, H. BIZZOZERONII, AND H. HEILMANNII S.S.

Only after successful *in vitro* isolation of these extremely fastidious microorganisms did pure bacterial isolates become available. The complete genomes of 4 of the 5 human-infecting *H. heilmannii* s.l. have now been published[80-83] (Table 2). The sequencing of these genomes showed that *H. suis*, *H. felis*, *H. bizzozeronii*, and *H. heilmannii* s.s. share many homologues to genes associated with colonization and virulence properties of *H. pylori* and of other bacteria[80-83]. These include the urease gene cluster, encoding a key enzyme to bacterial survival in the acidic gastric environment[6], the neutrophil-activating protein NapA, the γ-glutamyl transpeptidase, as well as complete or almost complete *comB* secretion system, required for DNA uptake by natural transformation[84]. Although these species contain homologues of genes encoding several outer membrane proteins of *H. pylori*, they do not harbor homologues to the BabA and SabA adhesins. They also lack homologues of the *H. pylori cag* pathogenicity island, including the gene encoding CagA, and of the vacuolating cytotoxin VacA. The *H. suis* genome is an exception, since it contains a *vacA* homologue[83]. The dissimilarities between the genomes of *H. heilmannii* s.l species and the *H. pylori* genome, including the lack of homologues to well-known *H. pylori* virulence factors associated with disease, may partially explain some of the differences between *H. pylori* and *H. heilmannii* s.l.-associated pathology[80-83].

Comparative genome analysis also provided a putative molecular basis for the zoonotic nature of *H. heilmannii* s.l species[85]. In comparison to *H. pylori*, *H. bizzozeronii*, *H. felis*, and *H. suis* have a higher metabolic versatility and a higher number of methyl-accepting chemotaxis proteins, possibly facilitating their adaptation and survival in the gastric environment of different host species[80,83,85].

PATHOGENESIS OF H. HEILMANNII S.L. INFECTIONS

The lack of pure isolates has also limited the information available on the pathogenesis and host responses of individual *H. heilmannii* s.l. species. The major exception is *H. felis* for which experimental models of infection have existed since the early 1990's, and for which different mice strains have been well-established as models of chronic gastritis[86], gastric atrophy[87-89], gastric MALT lymphoma[60], and gastric carcinoma[90,91]. Infection with *H. felis* in these models are often also used to study the pathogenesis of infection and the host immune response to *H. pylori*[92-95].

Table 2 General features of the available *Helicobacter heilmannii* s.l. species genomes and homology to *Helicobacter pylori* virulence genes

	H. suis		*H. felis*	*H. bizzozeronii*	*H. heilmannii* s.s.
Strain	HS1	HS5	CS1 (ATCC 49179)	CIII-1	ASB1
Host, Country	Pig, Belgium	Pig, Belgium	Adult cat, Australia	47-yr-old female patient with severe gastric symptoms, Finland	Kitten with severe gastritis, Belgium
Genome size (MB)	1635	1670	1673	1755	1805
G + C content	39.9%	39.9%	44.5%	46%	47.4%
CDSs	1266	1257	1671	1894	1918
Function assigned	1072	1066	1387	1280	1183
Plasmids	Not found	Not found	One (6.7 Kb; 5 CDSs)	One (52.1 Kb; 77 CDSs)	Not found
VacA	Yes (63% homology)	Yes (22% homology)	No	No	No
CagA	No[1]	No[1]	No	No	No
Ref.	Vermoote *et al*[83]		Arnold *et al*[80]	Schott *et al*[81]	Smet *et al*[82]

[1]Two members of the *cag* pathogenicity island (cag23/E and cagX) were identified in the *H. suis* genomes. CDSs: Coding sequences.

More recently, *H. suis*, *H. bizzozeronii*, and *H. heilmannii* s.s. pure isolates have been used in experimental models of infection[63,96-99]. Experimental infections with *H. suis* in Mongolian gerbils, BALB/c mice, and C57BL/6 mice showed that while in gerbils *H. suis* mainly colonized the antrum, in both mice strains *H. suis* was able to colonize the entire stomach[63]. Colonization with *H. suis* induced parietal cell necrosis in the 3 animal strains, epithelial cell hyperproliferation, and inflammation. *In vitro* data confirmed that *H. suis* causes apoptosis and necrosis of gastric epithelial cells, and indicated that the γ-glutamyl transpeptidase (GGT) virulence factor is involved in epithelial cell death[100]. *H. suis* GGT was also shown to inhibit T lymphocyte proliferation, and bacterial outer membrane vesicles were identified as a putative delivery route of GGT to the lymphocytes residing in the deeper mucosal layers[101].

Further experimental infections of BALB/c and C57BL/6 mice using 9 *H. suis* strains, showed that all *H. suis* isolates induced a predominant T-helper (Th)17 response, but only mild upregulation of the Th2 cytokine interleukin (IL)-4, and no upregulation of Th1 markers, including interferon (IFN)-γ[98]. This contrasts with previously published data which showed that *H. suis* induced a predominantly Th1 local immune response, and IFN-γ had a major role in the gastric inflammatory process[99,102]. A possible explanation for these differences is that previous experimental infection studies have used homogenized gastric specimens from mice, pigs or non-human primates instead of pure bacterial isolates[99,102].

In the Mongolian gerbil model, infection with *H. suis* led to the development of MALT lymphoma-like lesions in some animals[63], and in experimentally infected pigs, *H. suis* induced severe gastritis and a significant reduction in weight gain[103].

Concerning *H. bizzozeronii*, experimental infections in BALB/c, C57BL/6, SJL, and CFW mice showed that bacteria were mainly located in the gastric pits, dispersed through the mucous layer of the surface epithelium, or in close association with the parietal cells[104]. In the Mongolian gerbil model, *H. bizzozeronii* induced mild to moderate lymphocytic and neutrophilic infiltration in the gastric antrum of some animals, which was sometimes accompanied by mild parietal cell loss[105]. In the same study, transmission electron microscopy of *H. bizzozeronii*-infected gerbils showed neither necrotic parietal cells nor bacteria adhering to the epithelium[105]. Overall, *H. bizzozeronii* appears to be associated with a lower pathogenicity than *H. pylori* or *H. felis*[85].

Infection with 9 different *H. heilmannii* s.s. isolates in the Mongolian gerbil model showed that strains had different abilities to colonize the gerbil stomach. Furthermore, only 78% of the strains were able to induce chronic active gastritis and lymphocytic aggregation, caused by upregulation of IL-1β[96]. *H. heilmannii* s.s. strains with higher colonization ability were associated with higher fundic gastrin expression and reduced antral expression of the of H^+/K^+ ATPase pump[96].

Overall, these studies demonstrate that not only are there differences in the bacterium-host interactions between diverse *H. heilmannii* s.l. species, but there are also differences in the pathogenic potential in strains within the same species. Further studies will be necessary to address this question, namely the virulence factors involved and their putative associations with disease.

CONCLUSION

It is now recognized that *H. heilmannii* s.l. does not represent a single species, but rather several distinct *Helicobacter* species. *H. heilmannii* s.l. infect the stomach of several animals and may have zoonotic potential. Although the prevalence of these infections in humans is low, they are associated with gastric pathology and confer a higher risk of gastric MALT lymphoma than that of *H. pylori* infection, making them a significant health issue. So far, there are no studies that permit a clear stratification of the characteristics of the diseases according to each individual species that constitutes the group of gastric non-*H. pylori Helicobacter* species known as *H. heilmanni* s.l. Therefore, methods that allow bacterial identification at a species level are necessary to better clarify the prevalence of the infection in humans. Access to the full bacterial genome sequences together with the availability of in-

creasing number of *in vitro* isolates will allow significant advances in the understanding of bacteria-host interactions in disease pathogenesis and will be essential for developing strategies of prevention and treatment.

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REVIEW

Probiotics for antibiotic-associated diarrhea: Do we have a verdict?

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Abstract

Probiotics use has increased tremendously over the past ten years. This was coupled with a surge of data relating their importance in clinical practice. Antibiotic-associated diarrhea, whose frequency has risen recently, was one of the earliest targets with data published more than ten years ago. Unfortunately, available trials suffer from severe discrepancies associated with variability and heterogeneity of several factors. Most published randomized controlled trials and subsequent meta-analyses suggest benefit for probiotics in the prevention of antibiotic-associated diarrhea. The same seems to also apply when the data is examined for *Clostridium difficile*-associated colitis. However, the largest randomized double-blind placebo-controlled trial to date examining the use of a certain preparation of probiotics in antibiotic-associated diarrhea showed disappointing results, but it was flawed with several drawbacks. The commonest species of probiotics studied across most trials is *Lactobacillus*; however, other types have also shown similar benefit. Probiotics have enjoyed an impeccable safety reputation. Despite a few reports of severe infections sometimes leading to septicemia, most of the available trials confirm their harmless behavior and show similar adverse events compared to placebo. Since a consensus dictating its use is still lacking, it would be advisable at this point to suggest prophylactic use of probiotics to certain patients at risk for antibiotic-associated diarrhea or to those who suffered previous episodes.

Key words: Probiotics; Antibiotic-associated diarrhea; *Clostridium difficile*; Prevention; *Lactobacillus*; *Bifidobacterium*

Core tip: Probiotics use has been steadily increasing over the past ten years. One of the areas thoroughly examined includes prevention of antibiotic-associated diarrhea. Nonetheless, although trials are abundant, they are often confusing and conflicting. Adding insult to injury is the publication of the largest randomized controlled trial showing no benefit in prevention of antibiotic-associated diarrhea. We attempted to summarize, categorize and study the present literature detailing the important trials and their drawbacks in an attempt to come up with a reasonable consensus for their use.

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INTRODUCTION

Antibiotics use has been increasing steadily over the past decade; they are currently among the most prescribed medications worldwide. Their use elicits additional disturbances in the gut flora resulting in a multitude of symptoms at the clinical level. This ranges from mild diarrhea to electrolyte imbalance, sepsis, admission to the intensive care unit or even death[1]. Antibiotic-associated

Table 1 Most common trials in prevention of antibiotic-associated diarrhea/*Clostridium difficile*-associated diarrhea through probiotics

Study	Outcome	Population	Rx grp	Plb grp
Allen *et al*[41]	AAD/CDI	2941 in-patients	1470	1471
Armuzzi *et al*[29]	AAD	60 out-patients	30	30
Beausoleil *et al*[40]	AAD/CDI	89 in-patients	44	45
Beniwal *et al*[61]	AAD	202 in-patients	101	101
Can *et al*[25]	AAD/CDI	151 in-patients	73	78
Cimperman *et al*[23]	AAD	31 in-patients	15	16
Cremonini *et al*[30]	AAD	85 out-patients	22, 21, 21	21
Gao *et al*[38]	AAD/CDI	255 in-patients	86, 85	84
Gotz *et al*[28]	AAD	98 in-patients	48	50
Hickson *et al*[39]	AAD/CDI	135 in-patients	69	66
Lewis *et al*[21]	AAD/CDI	69 in-patients	33	36
McFarland *et al*[17]	AAD/CDI	193 in-patients	97	96
Myllyluoma *et al*[31]	AAD	47 out-patients	24	23
Nista *et al*[32]	AAD	120 out-patients	60	60
Pozzoni *et al*[22]	AAD/CDI	275 in-patients	141	134
Salminen *et al*[63]	AAD	17 out-patients (HIV)	9	8
Sampalis *et al*[18]	AAD/CDI	472 in-patients (ER)	233	239
Song *et al*[19]	AAD	214 in-patients	103	111
Stockenhuber *et al*[62]	AAD/CDI	678 in-patients	340	338
Surawicz *et al*[26]	AAD/CDI	318 in-patients	207	111
Thomas *et al*[20]	AAD/CDI	302 in-patients	152	150
Wenus *et al*[24]	AAD/CDI	87 in-patients	46	41
Wunderlich *et al*[27]	AAD	45 in-patients	23	22

AAD: Antibiotic-associated diarrhea; CDI: *Clostridium difficile* infect; HIV: Human immunodeficiency virus; ER: Emergency room.

diarrhea (AAD) is referred to as unexplained diarrhea that occurs in association with antibiotic administration[2]. Its incidence has been noted to slowly increase over the past few years, reaching up to 30% in some instances[3,4]. Symptoms can vary from mild self-limited disease to the more serious and severe *Clostridium difficile* (*C. difficile*)-associated diarrhea (CDAD). This issue may act as an important factor behind the non-adherence to antibiotic regimens[5]. Luckily, CDAD is only responsible for an estimated 10%-20% of cases of AAD[6]. Multiple risk factors for CDAD have been delineated, such as advanced age, hospitalization, acid suppression, chemotherapy, renal failure, gastrointestinal surgery and mechanical ventilation[3,7,8]. Reports from the United States have suggested a nearly 2-fold increase in mortality rate attributable to *Clostridium difficile* infect (CDI) diarrhea[9]. Another recent report from Canada has shown that regardless of the baseline above-mentioned risk factors, one out of every 10 patients who acquire *C. difficile* will die[10].

Probiotics were first reported more than 100 years ago and they were defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[11]. They have been thought to restore the disturbed gut flora through a multitude of mechanisms. They help reduce colonization of pathogenic organisms by competitively inhibiting their adhesion on the mucosa surface[12]. They have also been shown to secrete acids to decrease intraluminal pH, thus inhibiting the growth of several pathogens including enterohemorrhagic *Escherichia coli*[13,14]. They may also produce direct acting antimicrobial molecules[14]. Another proposed mechanism of action includes their immunomodulatory effect, which may diminish the inflammation caused by certain strains of bacteria[15]. Probiotics have become widely available in the market ranging from capsules to dairy food supplements stored in health stores and supermarkets. Their appeal lies in their availability and ease of intake as well as their low cost and low incidence of associated adverse events[16]. We conducted a literature review to assess the efficacy and safety of the use of probiotics in AAD in the adult population, and attempted to come up with a reasonable consensus for their use.

PROBIOTICS FOR THE PREVENTION OF AAD

The effectiveness of the use of probiotics in the prevention of AAD has been thoroughly examined in the past few years[17-28] (Table 1). Nonetheless, drawing conclusions from these publications has proven difficult secondary to a multitude of flaws, such as small numbers of patients, selection bias, vast heterogeneity in study populations, different probiotic types or dosage and sometime different end-points. Initially, several good quality randomized controlled trials (RCTs) with similar end-points showed a positive outcome on several variables including nausea, abdominal pain and diarrhea[29-32]. Two important meta-analyses were published in 2006, the first one included 25 RCTs and the second evaluated 16[33,34]. They both suggested that probiotics use was associated with a reduced risk of AAD. More recently, two large meta-analyses were released; the first by Videlock and Cremonini[35] in 2012 included studies with concurrent administration of probiotics and antibiotics. They analyzed 34 trials after exclusion and, with the use of a random effects model, they found a relative risk (RR) of AAD of 0.53 (95%CI: 0.44-0.63) when compared to placebo, their average number needed to treat (NNT) turned out to be 8 (95%CI: 7-11). Hempel *et al*[36] performed the second one the same year; this review included RCTs that evaluated probiotics as adjuncts to antibiotic use. Eighty-two trials met their inclusion criteria, of which 63 reported the number of patients with diarrhea, totaling 11811 participants. The RR to develop diarrhea compared with a control group was 0.58 (95%CI: 0.50-0.68). They also concluded a beneficial treatment effect with a NNT of 13. However, it is important to note that in this analysis RCTs were included only if probiotics were used to enhance the effect of antibiotics and therefore occurrence of diarrhea was not their primary end-point. A subgroup analysis involving only trials explicitly aiming to prevent or treat AAD showed similar results with an RR of 0.58 (95%CI: 0.49-0.68). Nonetheless, despite the fact that both these studies agreed there was sufficient evidence to support a preventive effect of probiotics supplementation on the incidence of AAD, they both suffered several limitations: lack of assessment of specific side effects, poor documentation of strains and of course large heterogeneity between the trials compared. A meta-

analysis published a few months ago aimed at drawing a better conclusion; they evaluated the efficacy of probiotics administered with antibiotics in reducing negatives outcomes[37]. They only included adult in-patients and excluded trials in which antibiotics were used for eradication of *Helicobacter pylori* as they were considered to represent a distinct clinical endpoint. They also discarded trials that were pilot studies of feasibility or tolerability because they did not define AAD incidence as an outcome, in addition to non-randomized comparisons or cohort studies. Due to their rigorous and strict inclusion criteria, they ended up with only 16 studies, all of which (except one) examined AAD as a primary outcome. Their meta-analysis demonstrated a statistically significant reduction in the risk of AAD with a RR of 0.61 (95%CI: 0.47-0.79), the NNT benefit was in the range of 11 (95%CI: 8-20). Their conclusion was favorable for probiotics in preventing AAD in the specific population of adult in-patients requiring antibiotics. The strength of their analysis was their policy of exclusive inclusion of trials with comparable outcome definition. Another was the focus on a specific target population thus decreasing heterogeneity between different publications. However, one significant limitation hindering most recent papers analyzing this issue is the surprising elevated rate of AAD found. In fact, three of the most recent RCTs reported rates as high as 34%-44%[38-40]. These high baseline event rates may have facilitated the detection of trends and significant outcomes despite small sample sizes. In general, most published papers agree to the benefit of probiotics in the context of AAD; however the largest RCT to date involving probiotics in the prevention of AAD failed to duplicate this result[41]. It is a multicenter randomized, double blind, placebo-controlled trial conducted by Allen *et al*[41] involving patients 65 years of age or older and exposed to at least one dose of antibiotics. They were randomized to either receive a preparation of Lactobacilli and Bifidobacteria totaling 6×10^{10} organisms, once per day for 3 wk or a placebo. Their primary outcome was assessment of the occurrence of AAD within 8 wk. They screened more than 17000 patients of which 1493 were assigned to the probiotics arm *vs* 1488 to the placebo group. Their results showed no difference in the occurrence of AAD between the two groups with an RR of 1.04 (95%CI: 0.84-1.28). Their conclusion stated that this multi-strain preparation showed no benefit in preventing AAD in this specific population. Although the methodology of this trial appears impeccable and the authors even tested the viability of their preparation before the intervention (often missed in other trials), it still displays several limitations. The first one was their low recruitment rate, which was less than one per five patients screened; the main reason being refusal to add an additional medication to their already large repertoire. Additionally, ethnic diversity in the study population was not ensured and this limits the generalizability of the conclusion already narrowed by the age group selection. Third, the rate of AAD occurring in both the probiotic and the placebo groups (10.8% and 10.4% respectively) is quite low compared to all the recent data. This is consistent with the diminishing trend in England[42] and Wales[43] but not with the rest of the world. Most importantly, their calculated sample size, which amounted to around 3000, was based on their assumption that the placebo group will have an AAD incidence of 20% and CDAD of 4%. However, their actual incidence rates turned out to be much lower than that, this obviously under-powers their end-result. All of the above arguments and drawbacks invite us to suspect bias and question the conclusion of this publication.

PROBIOTICS FOR THE PREVENTION OF CDAD

CDAD is considered a severe form of AAD; it usually affects 10%-20% of cases but some more recent studies have suggested that the actual figure may be closer to 30%[44,45]. CDI is a Gram positive, spore-forming rod that was first described in 1935 in newborn infants[46]. Exposure to antibiotics constitutes a definite risk factor for CDAD but also for asymptomatic CDI carriage[47]. Additionally, cumulative antibiotic exposure increases the risk[48,49]. Of great concern since 2003 has been an increased frequency and severity of CDAD associated with emergence of the hyper virulent 027 strain[50]. Recently, a large retrospective review involving more than 5600 patients reported that quinolone antibiotics have a stronger association with CDAD, whereas other antibiotics posed an intermediate risk[51]. Furthermore, a prospective cohort study involving 101796 admissions over a 5-year period at a tertiary care medical center classified antibiotics as high or low risk with relation to CDAD. They found that commonly used antibiotics like fluoroquinolones, cephalosporins, macrolides, clindamycin and carbapenems were among the high-risk group while all others were considered as low risk[52]. In addition to the multitude of risk factors for CDAD mentioned earlier, a recent variable has emerged over the past 3-4 years. Acid suppressive therapy has been suggested as an important risk factor for the development of CDAD[53]. According to Tal *et al*[54], an association between proton pump inhibitors (PPI) and CDAD is found with an odds ratio (OR) of 2.1 (95%CI: 1.2-3.5). Moreover, Barletta and colleagues reported in a retrospective case-control study that the probability for CDI was higher when PPI use exceeded 2 d in patients without prior hospital admission and 1 d in patients previously admitted[55]. The literature suggests that CDAD can occur after just one dose of antibiotics and may appear up to several weeks after completion of antibiotic therapy[56]. However, disease may progress despite antibiotic discontinuation and usually requires treatment with metronidazole or vancomycin. Considering that CDAD is a severe form of AAD, it seems imperative and clinically relevant to assess if probiotics can help in prevention.

The 2010 Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America

guidelines for the treatment of CDI do not recommend the use of probiotics for the prevention of CDAD due to lack of evidence and risk of blood stream infection[57]. Since this publication, there has been a multitude of newer studies in the literature examining the use of probiotics in the prevention of CDAD. The PLACIDE trial mentioned earlier also examined patients with CDAD and found the same disappointing results as for AAD. However, the events that occurred were somewhat low; 12 out of 1470 (0.8%) in the probiotic arm and 17 out of 1471 (1.2%) in the placebo group (OR = 0.7, 95%CI: 0.34-1.48)[41]. These figures raise a suspicious question since they are lower than most current numbers in the literature. In the subgroup analysis of their manuscript, Hempel *et al*[16] identified patients with CDI infection and showed that adjunct probiotic use extended the benefit to this severe section of patients as well with a RR of 0.52 (95%CI: 0.36-0.75). A large meta-analysis conducted in 2012 by Johnston *et al*[58] focused on probiotics in the prevention of CDAD. After their search and exclusion, they studied 20 RCTs deemed acceptable and included 1974 patients with positive CDI toxin *vs* 1844 placebo participants. They showed a large relative risk reduction in the incidence of CDAD of 66% corresponding to a RR of 0.34 (95%CI: 0.24-0.49). The authors concluded that there is moderate-quality evidence to support a protective effect of probiotics in the development of CDAD. This study failed, however, to reach its estimated optimal information size, which may have led to an overestimation of the beneficial role. In the 2013 review published by Pattani *et al*[37], they also assessed the effect of probiotics on the incidence of CDAD. Their analysis was inclusive of 9 RCTs and more than 1000 patients. The event rates were 18 (3.1%) of 572 patients in the intervention arm and 55 (10.4%) of 572 patients in the placebo arm, suggesting a RR of 0.37 (95%CI: 0.22-0.61). Their conclusion was that probiotics had a favorable impact in preventing CDAD in adult in-hospital patients.

FACTORS CONFOUNDING THE USE OF PROBIOTICS IN AAD

Several confusing factors hinder our understanding of probiotics and flaw the studies aiming to detect their beneficial effects. Perhaps the most complex one is the type and composition of various probiotics used. Should we use single or multiple strains in our prevention? Are certain strains more beneficial than others are? Johnston *et al*[58] addressed this issue in their review and found that trials using multiple species showed a larger effect (RR = 0.25, 95%CI: 0.15-0.41) than those using a single strain (RR = 0.5, 95%CI: 0.29-0.84) in preventing CDAD. The test for interaction suggested a low likelihood that chance alone explains such a difference (P = 0.06). They commented that the hypothesis is sufficiently credible to warrant further assessment through serious future studies[59].

Several strains of probiotics are currently available in the market, ranging from lactobacilli to bifidobacteria, saccharomyces, bacilli and others. When Pattani *et al*[37] pooled their studies by type of probiotic, reduction in AAD and CDAD persisted regardless whether a primarily *lactobacillus*-based probiotic or an *S. boulardii*-based formulation was used. The similarity in effect is reasonable and biologically plausible given that the benefit of probiotics is thought to derive (at least partly) from recolonization of the gastrointestinal tract with "normal", non-pathologic flora rather than from species-specific effect[60]. Hempel *et al*[36] were even more thorough in their analysis of different blends of probiotics genera. They found 17 RCTs with *Lactobacillus*-based interventions which showed a pooled RR of 0.64 (95%CI: 0.47-0.86) with a number needed to treat for benefit of 14. The 15 yeast-based (saccharomyces) RCTs revealed a pooled RR of 0.48 (95%CI: 0.35-0.65), NNT of 10. The results of three older studies involving *Enterococcus faecium* was a RR of 0.51 (95%CI: 0.38-0.68) and a NNT of 12. Hence, their analysis of different probiotic strains and types showed benefit across the board regardless of the genus or species.

Another conflicting factor is the age of the targeted population. In the PLACIDE trial, the authors could not find benefit in preventing both AAD and CDAD through their probiotics preparation in their adult 65 years and older patients[41]. They had chosen this particular age group because of their predilection to develop AAD[2,3]. Hempel *et al*[36] stratified the trials they studied according to age, they found 14 RCTs involving adults (age 18-60 years). The effect was found to be positive with a RR of 0.54 (95%CI: 0.34-0.85). On the other hand, three RCTs included exclusively elderly patients and the pooled result for these trials was a RR of 0.81 (95%CI: 0.40-1.63). These results are in accordance with the PLACIDE trial and suggest that probiotics use maybe beneficial in adults but not necessarily in the older age group. On another level, a further review of the literature showed an additional four RCTs (other than the PLACIDE) involving exclusively patients in the older age group[39,40,61,62]. All of these trials show statistically significant benefit in prevention of AAD by the probiotic group. The largest of these was performed in 2008 by Stockenhuber *et al*[62] and involved 678 patients aged 65 and above. It revealed a significant difference in the incidence of AAD between the placebo and the intervention group (17/340 *vs* 63/338). Compiling all the 5 RCTs together into one meta-analysis results in a large number of patients (4023) and shows a statistically significant difference in favor of the probiotic arm (Z = 3.58, P = 0.0003)[41]. However, despite limiting the scope of the studies involved, substantial statistical heterogeneity persists (P < 0.0001) and undermines any conclusion that can be drawn from it. No logical reasoning can explain this discrepancy; we can theorize that maybe physiological changes occurring with aging make the gastrointestinal tract less susceptible to the effects brought about by the alteration of gut flora.

It is very difficult to draw conclusions from the available data and meta-analysis regarding the duration of

treatment. The extent of heterogeneity between different studies precludes any reasonable analysis. This is also similar for the follow up period, as most publications do not precisely dwell on this issue.

SAFETY OF PROBIOTIC USE

Probiotics have enjoyed an impeccable reputation regarding safety. In general, little research attention has focused on adverse events in relation to their use in clinical practice[16]. This scarcity in data is partly a result of the Food and Drug Administration not regulating these products. One theoretical concern would be the potential transfer of antibiotic resistance, as many lactobacillus strains are naturally resistant to vancomycin. However, these resistance genes are chromosomal and not readily transferable to other pathogenic organisms[63]. Another theoretical risk would be the transfer of bacteria from the small intestine to other areas of the body, especially since infections suspected to be associated with the administered organisms were reported decades ago[16]. In some rare cases, probiotics have been linked to serious adverse effects such as fungemia and bacterial sepsis[64-70]. Few risk factors have been identified through these case reports and they include severe immune-suppression or infant prematurity. Additional factors have been shown to include insertion of central venous catheter, short gut syndrome, cardiac valvular heart disease or the presence of a jejunostomy tube[71]. An alarming study published in 2008 aimed at examining the effect of probiotics in hospitalized patients with a predicted severe acute pancreatitis[72]. Not only did they fail to show any benefit regarding infectious complications in the probiotic arm but also they additionally revealed a statistically significant increase in mortality and an increased risk of bowel ischemia compared to placebo. They concluded that physicians should be careful in their use of probiotics, especially in severely sick patients.

Examining available data for adverse events of probiotics is not an easy task; it is mostly under-reported in the literature. In their trial, Allen *et al*[41] found a statistically significant difference in flatus in the probiotic group. Almost 20% of participants had serious adverse events, but the frequency was similar in both groups. The most common were respiratory, mediastinal and thoracic disorders (5.9%). In the 2012 review performed by Johnston *et al*[58], 17 RCTs reporting on side effects were assessed[55]. Four reported no adverse events at all and three reported serious ones. However, the frequency of events was higher in the control group (12.6% *vs* 9.3%). The most commonly reported symptoms were abdominal cramping, nausea, fever, soft stools and flatulence. When Pattani *et al*[37] performed their meta-analysis they found no life threatening adverse effects in the 16 RCTs studied. Furthermore, one of the largest meta-analyses to-date assessing probiotics is the one performed by Hempel *et al*[36] in 2012; it included 84 RCTs, of which 59 did not report on probiotic-specific adverse events. The rest did not mention any serious side effects. More importantly, three recent systematic reviews have addressed the safety of probiotics[16,73,74]. The most comprehensive of them[16] searched 12 electronic databases; they included 208 RCTs. For short-term probiotic use compared with the control group there was no statistically significant difference in the overall number of adverse events (RR = 1.00, 95%CI: 0.93-1.07) including serious ones (RR = 1.06, 95%CI: 0.97-1.16).

CONCLUSION

A substantial number of trials have been published examining the use of probiotics in the prevention of AAD. However, few of these were adequately powered enough to demonstrate a reduction in a relatively rare event (< 15%). Associations were shown and conclusions drawn through pooling results across inadequately powered RCTs. Several variables are still unclear in their interactions with probiotics. We have isolated only few RCTs exclusive to elderly patients, therefore potentially important but unknown factors might include the characteristics of the pre-treatment enteric flora, which varies between individuals and is affected by age. Additionally, the strain, dose and duration of probiotics used in the various studies vary widely, therefore making it difficult to draw strong conclusions regarding probiotic use. There are still many unanswered questions to be tackled by larger RCTs, such as: which patient population will benefit the most from probiotic supplementation; which probiotic strains are most effective and does this efficacy vary with the clinical indication or the dose; and finally what are the real risks and hazards associated with routine use of such medications.

The appeal of using probiotics comes clearly from their ready availability, low cost and acceptable known safety profile. With the current data at hand, it is difficult to draw any solid conclusion about the prophylactic use of probiotics in AAD. It would be reasonable to advise their use in some specific populations such as patients with a history of AAD or risk factors for the development of CDAD. Many physicians have been hesitant to adopt probiotics in their routine practice; it would be advisable at this point to stratify this use on case-by-case basis.

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REVIEW

Molecular diagnosis and therapy for occult peritoneal metastasis in gastric cancer patients

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Abstract

To apply an individualized oncological approach to gastric cancer patients, the accurate diagnosis of disease entities is required. Peritoneal metastasis is the most frequent mode of metastasis in gastric cancer, and the tumor-node-metastasis classification includes cytological detection of intraperitoneal cancer cells as part of the staging process, denoting metastatic disease. The accuracy of cytological diagnosis leaves room for improvement; therefore, highly sensitive molecular diagnostics, such as an enzyme immunoassay, reverse transcription polymerase chain reaction, and virus-guided imaging, have been developed to detect minute cancer cells in the peritoneal cavity. Molecular targeting therapy has also been spun off from basic research in the past decade. Although conventional cytology is still the mainstay, novel approaches could serve as practical complementary diagnostics to cytology in near future.

Key words: Gastric cancer; Peritoneal lavage; Cytology; Molecular diagnostic techniques; Reverse transcriptase polymerase chain reaction; Carcinoembryonic antigen

Core tip: For patients with gastric cancer, cytological detection of cancer cells in the peritoneal cavity is important to predict future manifestation of peritoneal recurrence. However, its improvement has been a matter of research, because of its low sensitivity and specificity. The new diagnostic modalities have been investigated along with the development of modern molecular biology. The recent innovative challenges regarding molecular diagnosis of intra-peritoneal gastric cancer cells have been thoroughly covered and summarized. The new therapies for gastric cancer with peritoneal spreads were also referred.

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INTRODUCTION

Gastric cancer is one the leading causes of death in the world[1], and the most prevalent cancer in Eastern Asia[2]. Although the radical resection of cancerous lesions is the only cure for gastric cancer, multi-disciplinary therapy

for advanced disease can palliate the disease and even prolong life[3,4]. Therefore, the accurate and appropriate diagnosis of the disease entity is required so that an individualized oncological approach can be used. The tumor-node-metastasis (TNM) staging system is the universally accepted method to describe the degree of cancer advancement[5,6]. As with other cancers, gastric cancer has disease-specific factors in its staging. One of them is the cytology of a peritoneal wash or ascites because peritoneal metastasis is the most frequent mode of distant metastasis and post-surgical recurrence. However, it is often difficult to diagnose peritoneal metastasis by conventional imaging modalities, such as computed tomography and positron emission tomography. The cytological detection of free cancer cells in the peritoneal cavity is a very important finding in gastric cancer. Positive cytology means that peritoneal metastasis exists anywhere in the peritoneal cavity even if it is invisible, so it implies a high probability of future manifestations of peritoneal metastasis[7-12]. Therefore, peritoneal lavage cytology findings as well as peritoneal metastasis are factors in gastric cancer staging in Japan as stage 4 disease[13]. The most recent TNM classification system includes intraperitoneal cancer cell detection as part of the staging process, denoting metastatic disease[5].

Peritoneal carcinomatosis is an incurable disease with poor prognosis. In cases of peritoneal carcinomatosis, although debate about surgical application still remains, palliative chemotherapy would be preferred[14-17]. From this point of view, peritoneal carcinomatosis needs to be precisely diagnosed before surgery or at the beginning of surgery for surgeons to determine the most appropriate therapeutic approach[18]. However, in reality, the uneven shape of the peritoneal cavity makes it impossible for the entire cavity to be thoroughly inspected and difficult for the surgeon to definitively judge whether the peritoneal cavity is completely free of metastatic foci. Consequently, peritoneal lavage cytology is needed for the indirect diagnosis or prediction of peritoneal metastasis, and it must be as accurate as possible. The accuracy in peritoneal lavage cytology depends greatly upon the experience of the cytopathologist; therefore, the diagnosis remains inevitably subjective. In addition, several studies indicate that the sensitivity and specificity of peritoneal lavage cytology is unsatisfactory and that there is still room for improvement[19]. Over the past decade, several new diagnostic approaches have been studied. As an alternative to conventional cytology by Papanicolaou staining, immunocytochemistry or PCR-based genetic detection of epithelial or malignant cells in the peritoneal fluid has emerged (Table 1). There are advantages and shortcomings of each approach[20]. In this review, we examine recent studies, summarize findings on the molecular biology-based diagnosis of peritoneal cancer cell existence, and discuss recent advances in the treatment of peritoneal carcinomatosis.

CONVENTIONAL CYTOLOGY

Since the method of lavage cytology was described by Moore *et al*[21] in 1961, several clinical studies have demonstrated the prognostic significance of intraperitoneal free cancer cells at the time of surgery[7,10,12,16,17,22-25]. The Japanese Classification of Gastric Carcinoma (2nd English edition) first included the result of peritoneal cytology as one of the staging parameters in 1999[26]; since then, the Japanese Gastric Cancer Association includes peritoneal cytology in their staging system[14]. Although the most recent TNM classification has included the detection of intraperitoneal free cancer cells as part of the staging process, denoting M1 disease[5], the application of peritoneal cytology in preoperative staging is still controversial. The European Society for Medical Oncology practice guidelines recommend laparoscopy, but regard cytology as optional, and the current National Comprehensive Cancer Network (NCCN) guidelines also do not include cytology in the treatment algorithm[27]. Nevertheless, peritoneal cytology has important clinical implications in the management of advanced gastric cancer[7,28].

In gastric cancer surgery, by either laparotomy or laparoscopic approach, about 100-200 mL of saline is usually instilled into the Douglas pouch (and occasionally into the left subphrenic space) and gently stirred. A washing sample is then aspirated and subjected to cytology. Traditionally, Papanicolaou or Giemsa stainings are employed, and specimens are diagnosed by experienced cytopathologists. The accuracy, sensitivity, and specificity of conventional cytology in predicting peritoneal recurrence was 73.0%-91.9%, 11.1%-80.0%, and 86.4%-100.0%, respectively[20]. Thus, sensitivity had a particularly wide range, which indicated the need for further advanced techniques.

CARCINOEMBRYONIC ANTIGEN IN PERITONEAL LAVAGE

Kanetaka *et al*[29] recently reported that the measurement of carcinoembryonic antigen (CEA) level in peritoneal lavage (pCEA) by an enzyme immunoassay can predict poor prognosis and may help to elucidate a cohort who need more intensive adjuvant chemotherapy to improve their prognosis. Since Asao *et al*[30] first reported that the CEA antigen level in peritoneal lavage could reflect the presence of peritoneal metastasis more accurately than conventional cytology in 1991, other investigators have demonstrated the clinical significance of pCEA levels[31-35]. Most of these reports showed a significant correlation between pCEA level and survival after surgery, implying that pCEA could be a potential predictor of poor prognosis. However, the pCEA level may reflect both the production of CEA in the peritoneal cavity and the serum CEA level and may not be specific as a marker for the existence of intraperitoneal free cancer cells or occult peritoneal metastasis.

Table 1 List of published studies regarding the molecular diagnosis of peritoneal fluid in gastric cancer

Ref.	Molecule	Technique	Number of patients	Results
Asao *et al*[30]	CEA	Enzyme immunoassay	120	Correlation with 2-yr survival rate
Irinoda *et al*[32]	CEA, sialyl-Tn antigen	Enzyme immunoassay	96	Correlation with peritoneal metastasis and prognosis
Abe *et al*[31]	CEA	Enzyme immunoassay	56	Correlation with peritoneal metastasis and overall survival
Cetin *et al*[34]	CEA	Enzyme immunoassay	70	Correlation with peritoneal metastasis and overall survival
Kanetaka *et al*[29]	CEA	Enzyme immunoassay	597	Correlation with overall survival and peritoneal recurrence free survival
Yamamoto *et al*[33]	CEA, CA125	Enzyme immunoassay	229	Correlation with overall survival and recurrent cites
Li *et al*[35]	CEA	Radioimmunoassay	64	Correlation with overall survival
Kodera *et al*[38]	CEA	RT-PCR	189	Correlation with overall survival and peritoneal recurrence-free survival
Wang *et al*[36]	CEA	RT-PCR	40	Correlation with peritoneal recurrence
Sugita *et al*[41]	CEA, CK20	RT-PCR	129	Correlation with overall survival and peritoneal recurrence-free survival
Dalal *et al*[37]	CEA, CK20, survivin, MUC2	RT-PCR	40	CEA had high sensitivity and specificity, while CK20, survivin, and MUC2 showed high false-positive rates
Takata *et al*[42]	CEA, CK20	RT-PCR	104	Predict peritoneal recurrence
Kodera *et al*[40]	CK20	RT-PCR	195	Not sufficiently sensitive as CEA
Yonemura *et al*[39]	MMP-7	RT-PCR	152	Improved the sensitivity for peritoneal dissemination in combination with cytology
Mori *et al*[43]	Multiple marker	Microarray	179	Correlation with disease-free survival and immuno-cytochemical cytology
Hiraki *et al*[52]	Aberrant gene methylation	Methylation-specific PCR	107	Correlation between positive methylation and peritoneal recurrence
Mori *et al*[56]	Telomerase activity	TRAP assay	46	Some concordance with cytology
Da *et al*[57]	Telomerase activity	TRAP assay	60	Correlation with high proliferating activity of gastric cancer
Wong *et al*[62]	Viral tropism	NDV-GFP imaging	30	Higher sensitivity and lower specificity than cytology
Kitayama *et al*[58]	EpCAM	Flow cytometry	195	Tumor cell/leukocyte ratio reflects peritoneal spread

CEA: Carcino-embryonic antigen; CA125: Cancer antigen 125; CK20: Cytokeratin 20; TRAP assay: Telomeric repeat amplification protocol assay; NDV-GFP: Newcastle disease virus-green fluorescent protein; MUC2: Mucin 2; RT-PCR: Reverse transcription polymerase chain reaction.

GENETIC DETECTION OF INTRAPERITONEAL GASTRIC CANCER CELLS

Molecular diagnosis with reverse transcriptase-polymerase chain reaction (RT-PCR) has been employed for the detection of minimal cancer cells due to its high sensitivity. Among the messenger RNA (mRNA) specific to cancer cells or epithelial cells, the most common target molecule is CEA mRNA. PCR evaluation of CEA mRNA in peritoneal fluid has increased sensitivity for the detection of peritoneal cancer cells as compared to cytology[36,37], and positive results have been associated with poor survival. Kodera *et al*[38] demonstrated that CEA PCR-positive patients had significantly worse overall survival and recurrence-free survival as compared to PCR-negative patients, independently of cytology. PCR appears to increase the accuracy of detection of occult disease.

In addition, molecular targets for PCR other than CEA have been investigated and include metalloprotease-7[39] and cytokeratin 20[40,41]. The expression level of a single gene was heterogeneous, so limited sensitivity hinders its use alone. To further improve the sensitivity and specificity of the mRNA detection approach, multiplex PCR may prove to be more clinically useful in capturing intraperitoneal free cancer cells[41-43].

Mori *et al*[44] tried to select marker candidates out of tens of thousands of genes with microarray analysis, and they identified the genes specific to cytology-positive samples. They further manufactured a microarray chip containing 10 marker genes as a "MiniChip" and demonstrated that the MiniChip assay has a sensitivity and specificity equal to or better than conventional cytology in detecting minimal free cancer cells in peritoneal fluid[43].

Recently, a new rapid genetic diagnostic technique to detect minute cancer cells has been developed and applied in the sentinel node navigation surgery as surgical decision making[45-48]. One-step nucleic acid amplification (OSNA) uses reverse transcription loop-mediated isothermal amplification (RT-LAMP) to detect mRNA expression of target sequences from crude samples without RNA purification[49]. The reaction can be completed in a single test tube and within 1 h. Kumagai *et al*[50] reported a multicenter study evaluating the clinical performance of the OSNA assay that detects cytokeratin 19 (CK19) mRNA in detecting lymph node (LN) metastases in gastric cancer patients, and this method showed high concordance rate to pathology. Although the OSNA assay is useful in the intraoperative rapid diagnosis of LN metastasis for gastric cancer, it remains unproven if this technique could be ap-

plied to detect intra-peritoneal free cancer cells. It needs to be determined how the different properties of cells in the peritoneal cavity interfere with the reaction and what the minimal number of cancer cells is for detection by this method.

DNA methylation is an important epigenetic change in cancer that leads to the recruitment of transcription repressors and chromatin changes, so methylation analysis has been used as a diagnostic modality for various cancers[51]. Hiraki *et al*[52,53] assessed whether gene methylation in peritoneal fluid from gastric cancer patients is clinically feasible for determining the peritoneal metastasis in gastric cancer. By using quantitative methylation-specific PCR to compare aberrant methylation status in gastric cancer, they isolated 6 genes (*BNIP3*, *CHFR*, *CYP1B1*, *MINT25*, *RASSF2* and *SFRP2*) as having cancer-specific DNA methylation, and they observed that there was a significant correlation between positive methylation in any of these 6 genes and peritoneal recurrence[52]. Thus, methylation analysis might improve the positive detection of gastric cancer cells in peritoneal lavage.

TELOMERASE ACTIVITY IN THE PERITONEAL FLUID

Telomerase activity in cancer cells has been examined as a tag to detect cancer cells in the peritoneal cavity. Telomerase activity is one of the hallmarks of cancer and can be used to discriminate malignant cells from normal ones[54,55]. Mori *et al*[56] analyzed peritoneal lavage fluid employing a TRAP assay that reflects telomerase activity. To improve the efficacy of the assay, they enriched cancer cells with immunomagnetic beads coated with anti-Ber-EP4 antibody. Then, they successfully detected telomerase activity in the samples from gastric cancer patients with serosal or subserosal invasions, and they found some concordance with the results of cytology[56]. Da *et al*[57] have also investigated the telomerase activity in peritoneal lavage from gastric cancer patients without enrichment of cancer cells. Although the sample size was relatively small, their data demonstrated that all patients with peritoneal metastasis had detectable telomerase activity in peritoneal lavage fluid, and they found significant correlations between positive rate of telomerase activity and invasion depth, serosa-involved areas, and the presence and extent of peritoneal metastasis. While these methods were unique and appeared to be sensitive, they were not significantly superior to conventional cytology by itself. Nevertheless, telomerase activity analysis in peritoneal lavage fluid might be a helpful adjunct for the cytology in the diagnosis of occult peritoneal metastasis of gastric cancer.

FLOW CYTOMETRIC ANALYSIS OF FREE CANCER CELLS IN PERITONEAL LAVAGE FLUID

Kitayama *et al*[58] tried to quantify the free cancer cells recovered from ascites or peritoneal lavage fluid from gastric cancer patients by conventional flow cytometry. The peritoneal lavage fluid from gastric cancer patients contains erythrocytes, leukocytes, dissociated peritoneal mesothelium, and a small number of cancer cells. Therefore, molecular detection needs to distinguish cancer cells from normal cells co-existing in the peritoneal cavity. Kitayama *et al*[58] stained the cells with monoclonal antibodies to CD45 and CD326 (EpCAM), and CD326-positive and CD45-positive cells were classified as either cancer cell or leukocytes. Instead of using the total number of cancer cells, they calculated the cancer cell/leukocyte ratio and demonstrated that the ratio was significantly higher in the patients with peritoneal metastasis and positive cytology than in those without peritoneal spread. They further showed the ratio to reflect well the effect of intraperitoneal chemotherapy. They thus proposed that the flow cytometry-based measurement of the intraperitoneal CD326(+)/CD45(+) ratio could be a diagnostic marker that reflects the severity of peritoneal metastasis as well as the effectiveness of intraperitoneal chemotherapy.

Besides gastric cancer, ovarian cancer also often forms excess ascites due to peritoneal metastasis, which is routinely drained and discarded for symptomatic relief. Peterson *et al*[59] regard the ascites as a source of cancer cells for monitoring the treatment response of ovarian cancer. Miniaturizing and advancing flow cytometric technology, they developed and tested a new microfluidic chip to capture, enrich and analyze ascites tumor cells in ovarian cancer patients. This technology allows the detection of occult cancer cells and enables the molecular profiling of individual cells. The microfluidic chip might be applicable to the diagnostic and molecular analysis of peritoneal fluid from gastric cancer patients.

DIAGNOSTIC POTENTIAL OF THE VISUAL DETECTION OF CANCER CELLS IN PERITONEAL CYTOLOGY SAMPLES

As a unique approach, several groups examined virus-mediated fluorescent gene expression to visually detect rare cancer cells in the body fluid or the cytology samples against millions of normal cells[55,60,61]. Wong *et al*[62] evaluated a novel detection technique for intraperitoneal free cancer cells by using Newcastle disease virus-green fluorescent protein (NDV-GFP), which is genetically modified NDV that expresses the green fluorescent protein gene. Newcastle disease virus has been studied since the 1950s for its ability to infect and replicate specifically in tumors. NDV-GFP targets and infects specifically cancer cells, resulting in specific GFP expression. Wong *et al*[62] evaluated peritoneal lavage samples from 30 gastric cancer patients undergoing staging laparoscopy with NDV-GFP. They found that NDV-GFP-mediated detection offers a more sensitive method of identifying free peritoneal gastric cancer cells in peritoneal lavage fluid as compared to conventional Pap staining cytology to dem-

onstrate that NDV-GFP could be used diagnostically.

WHAT IS NEXT FOR THE IMPROVEMENT OF INTRAPERITONEAL DIAGNOSIS?

As described above, numerous efforts have been made to improve the detection of intraperitoneal free cancer cells. The purpose of most of these studies appeared to primarily be an improvement of the accuracy in cytology. The secondary purpose will be to make diagnosis more convenient and automatic than subjective conventional cytology. Once the accuracy and procedure is essentially improved over the conventional cytology, what should we do next? The identification of intraperitoneal free cancer cells confers poor prognosis. In patients with positive cytology without macroscopic peritoneal metastasis, the benefit of radical or aggressive surgery is still a matter of debate. While some of these patients are palliated, others may undergo more aggressive therapies. Along with the improved diagnostic modality, the treatment strategy would also have to be a coupled issue.

MULTIMODAL CLINICAL APPROACH FOR PERITONEAL SPREAD OF GASTRIC CANCER

Surgeons have witnessed some patients with peritoneal spread of gastric cancer who underwent radical surgery and experienced cures due to the recent improvements in multimodal treatment. A phase Ⅱ study of whether gastrectomy with curative intent would be beneficial for patients with positive cytology but absence of macroscopic peritoneal seeding has been conducted[63,64]. The study showed that median overall survival time was 705 d, and the 5-year survival rate was 26% in the patients with positive cytology with no other non-curative factors, suggesting that surgery with curative intent could be indicated even for patients with positive cytology[63,64]. For gastric cancer patients with macroscopic peritoneal metastasis, Yamaguchi *et al*[65] evaluated intraperitoneal chemotherapy along with systemic chemotherapy as a phase Ⅱ study. They reported a 1-year survival rate of 77.1%, which is surprisingly high. The same group also reported salvage gastrectomy after intravenous and intraperitoneal chemotherapy for the patients who had peritoneal metastasis but showed apparent shrinkage of their peritoneal nodules as well as negative cytology by the treatment[66]. Those patients who underwent salvage gastrectomy exhibited a 26.4-mo median survival period and 82% of 1-year overall survival. Those results suggested that the more sensitive and specific peritoneal diagnosis with the molecular approach might allow gastric cancer patients to receive more suitable individualized multimodal therapies.

A NEW MOLECULAR-TARGETING THERAPY FOR INTRAPERITONEAL SPREAD OF GASTRIC CANCER

Along with the research for the improvement in detection of intraperitoneal cancer cells, molecular targeting therapies might be derived from the results of basic research. One of the molecular targets is epithelial cell adhesion molecule (EpCAM), a type I transmembrane glycoprotein functioning as a homotypic intercellular adhesion molecule[67]. High-level EpCAM expression was observed in 90.7% of gastric cancer[68]. Catumaxomab is an artificially engineered, tri-functional bispecific monoclonal antibody; Fab binding sites bind to EpCAM on cancer cells and CD3 on T cells, and the Fc region binds and activates accessory immune cells. The tri-cell complex of T-cells, tumor cells and accessory cells induces MHC-unrestricted but specific efficient tumor cell killing. The therapeutic benefit of Catumaxomab for patients with malignant ascites including gastric cancer patients has been reported in a pivotal clinical trial[69], which led to approval of Catumaxomab by the European Medicines Agency (EMA) in 2009. Intraperitoneal Catumaxomab treatment has been shown to trigger the activation of immune effector cells in the peritoneal cavity resulting in the depletion of EpCAM-positive tumor cells[70]. Thus, local strategies with molecular targeting agents might represent the appropriate option for treatment of the peritoneal spread of gastric cancer.

CONCLUSION

In the past decade, enormous strides have been made in the research for molecular detection of intraperitoneal free gastric cancer cells, and many new strategies have been clinically tested in gastric cancer patients. As with the conventional cytology, none of the candidate alternatives to conventional cytology are a perfect modality yet, whereas most of them would potentially be conducive to improve the conventional diagnosis and to predict prognosis. The uncertainty of a definition of positivity in these novel approaches and their clinical relevance remain potential limitations to the practical clinical use of these technologies. Too highly sensitive techniques such as PCR may result in the detection of clinically irrelevant metastatic disease, which could lead to either overtreatment with unnecessary chemotherapy, or worse, the withdrawal of potentially curative surgical treatment. Nevertheless, the development of more sensitive and rapid diagnostics in evaluating minimal peritoneal disease is needed for patients to be properly treated. Since peritoneal lavage cytology has recently been included in the staging criteria of gastric cancer, the cytology diagnosis has been focused on as having an important predictive

role in gastric cancer treatment, and the molecular diagnosis has undergone tremendous challenges. With the accumulated evidence, the molecular diagnosis of peritoneal cytology may be a reality in future gastric cancer practice.

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REVIEW

Cancer-associated fibroblasts in digestive tumors

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Abstract

The significant influence of tumor stroma on malignant cells has been extensively investigated in this era of targeted therapy. The tumor microenvironment, as a dynamic system, is orchestrated by various cells including tumor vascular composing cells, inflammatory cells and fibroblasts. As a major and important component in tumor stroma, increasing evidence has shown that spindle-shaped cancer-associated fibroblasts (CAFs) are a significant modifier of cancer evolution, and promote tumorigenesis, tumor invasion and metastasis by stimulating angiogenesis, malignant cell survival, epithelial-mesenchymal transition (EMT) and proliferation *via* direct cell-to-cell contact or secretion of soluble factors in most digestive solid tumors. CAFs are thought to be activated, characterized by the expression of α-smooth muscle actin, fibroblast activated protein, fibroblast specific protein, vimentin, fibronectin, *etc*. They are hypothesized to originate from normal or aged fibroblasts, bone marrow-derived mesenchymal cells, or vascular endothelial cells. EMT may also be an important process generating CAFs, and most probably, CAFs may originate from multiple cells. A close link exists between EMT, tumor stem cells, and chemo-resistance of tumor cells, which is largely orchestrated by CAFs. CAFs significantly induce immunosuppression, and may be a prognostic marker in various malignancies. Targeted therapy toward CAFs has displayed promising anticancer efficacy, which further reinforces the necessity to explore the relationship between CAFs and their hosts.

Key words: Cancer-associated fibroblast; Tumor progression; Epithelial-mesenchymal transition; Tumor immunity; Targeted therapy

Core tip: As a dominant component in tumor stroma, cancer-associated fibroblasts (CAFs) promote tumorigenesis, and tumor progression by stimulating angiogenesis, malignant cell survival, epithelial-mesenchymal transition (EMT) and proliferation *via* direct cell-to-cell contact or secretion of soluble factors in most digestive solid tumors. CAFs are characterized by the expression of α-smooth muscle actin, fibroblast activated protein, fibroblast specific protein, vimentin, *etc*. They are hypothesized to originate from various cells. EMT may also be an important process generating CAFs. A close link exists between CAFs-induced EMT, chemo-resistance of tumor cells, and tumor stem cells. CAFs significantly induce immunosuppression, and may be a prognostic marker. Targeted therapy toward CAFs has displayed promising anticancer efficacy.

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INTRODUCTION

Tumors are made up of heterogeneous cells, and are considered to be wounds that do not heal[1]. Carcinogenesis and tumor progression are not only associated with malignant cells themselves, but also with tumor stroma which is an important participant and regulator. Both interact with each other, forming the tumor-host microenvironment[2]. In tumor stroma there are various types of mesenchymal cells including tumor vascular composing cells (endothelial cells and pericytes), inflammatory cells and fibroblasts, which are mosaicked in extracellular matrix (ECM) secreted by fibroblasts[3]. Stromal cells provide malignant cells with growth and proliferation signals, and participate in angiogenesis within tumor. Tumor cells are to stromal cells what "seeds" are to "soil". The remarkable influence of tumor stroma on cancer cells has been extensively investigated in this era of targeted therapy.

Cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs) are the most abundant with the widest distribution in tumor stroma of most solid tumors, and one of the most important stromal cells mediating tumor-stroma cross-talk (Figure 1). It can not only directly act on malignant cells in a direct cell-to-cell way and by secreting diverse soluble cytokines, regulating tumor evolution, growth, angiogenesis, immunity, chemoresistance, and aggressive behaviors (invasion and metastasis)[4-6], but also interfere with tumor growth indirectly by influencing other stromal components including ECM (Figure 2)[7]. Researchers have proposed the CAFs-centered theory, thinking that CAFs are the hub and control center of tumorigenesis and tumor progression[8]. In recent years, CAFs have become a focus of research, with the hope that they'll be a novel anti-tumor therapeutic target.

DEFINITION AND CHARACTERISTICS OF CAFS

Carcinogenesis resembles injury repair in inflammatory cell infiltration, angiogenesis, fibrogenesis, and fibroblast activation[7]. CAFs were initially identified in tumor stroma of solid tumors with connective tissue generation including breast cancer and pancreatic cancer, and are highly homologous large spindle-like stromal cells with the ability to adhere to plastic[9]. Fibroblasts are versatile, plastic cells that can respond to environmental signals through diverse functional programs. Compared with fibroblasts in normal wound healing, they are fibroblasts with activated phenotypes, which grants CAFs multiple functions in promoting cancer development[10], and share a similar formation process with myofibroblasts[11]. CAFs are characterized by CD34 expression deletion, and abundant expression of α-smooth muscle actin (α-SMA)[11,12]. CAFs also express fibroblast activated protein (FAP), fibroblast specific protein (FSP), neuron glial antigen-2 (NG2), platelet derived growth factor receptor-α (PDGFR-α) and PDGFR-β. Like normal fibroblasts, stromal cell markers including vimentin, fibronectin, podoplanin, prolyl 4-hydroxylase (P4H, 5B5) and stromelysin are also expressed[13].

Research has revealed that paracancerous fibroblasts grow much faster than CAFs segregated and cultured *in vitro*[14], suggesting that the proliferative ability of CAFs are closely linked with the specific tumor microenvironment, and that CAFs may be activated by various malignant cell-secreted cytokines. In prostate cancer, interleukin (IL)-6 secreted by malignant cells was found to contribute to the formation of CAFs[15]; in hepatocellular carcinoma, lysophostatidic acid (LPA) was reported to transform paracancerous fibroblasts into CAFs[16]; transforming growth factor-β (TGF-β) and IL-1β were also revealed to induce the expression of α-SMA and FAP in normal fibroblasts. Changes in cellular genome, such as global hypomethylation of genomic DNA may also be involved in activation of CAFs[17].

CAFs can synthesize and secrete abundant soluble molecules containing the fibroblast growth factor family such as basic fibroblast growth factor (bFGF), the vascular endothelial growth factor (VEGF) family members, platelet-derived growth factor (PDGF), ligands of epidermal growth factor receptor (EGFR), the IL family members including IL-1, the colony-stimulating factors including granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage CSF (GM-CSF), and TGF-β, whose major functions in tumor are breaking the balance of the microenvironment to induce stromal reaction, stimulating angiogenesis *via* a paracrine, mediating inflammatory reaction and activating surrounding stromal cells to secrete cytokines or proteinases, which resembles wound healing[18,19]. CAFs are therefore also called myofibroblasts in tumor stroma, paracancerous fibroblasts or tumor-derived fibroblasts[20].

ORIGINS OF CAFS

The origin of CAFs is still obscure[21]. CAFs are considered to derive from various cells. Fibroblasts in tumor stroma are hypothesized to be the origin because fibroblasts in metastatic foci of colorectal carcinoma share the same protein labeling with the resident fibroblasts[22]. Particularly in hepatocellular carcinoma (HCC) which is usually derived from chronic hepatitis and liver cirrhosis, large amounts of static fibroblasts are activated after repeated inflammatory stimulation, injury repair, and tissue fibrosis, forming a large number of CAFs. The expression of α-SMA in hepatic stellate cells (HSCs) increases gradually indicating their activation, and suggesting them as a possible origin in HCC[23]. Fibroblasts in peritumoral tissue progressively convert into CAFs during the course of tumor progression, and LPA can accelerate HCC progression by recruiting peri-tumor fibroblasts (PTFs) and promoting their transdifferentiation into myofibroblasts[16,24]. FSP and

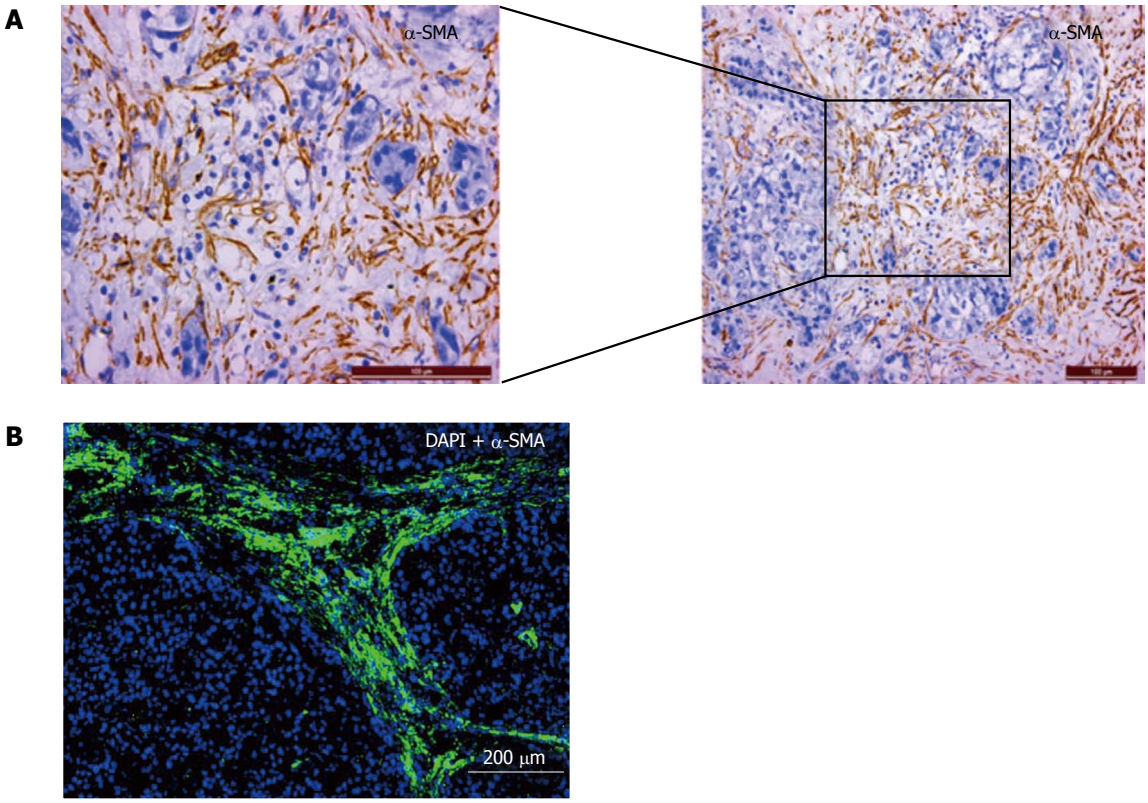

Figure 1 Hepatocellular carcinoma-associated cancer-associated fibroblasts and hepatocellular carcinoma cells. A: The distribution of H-CAFs identified by α-SMA (+) expression in a HCC specimen is detected by immunohistochemistry. Expression of α-SMA (shown in brown color) is detected to confirm the presence of H-CAFs, which are abundant in tumor tissue; B: The presence of H-CAFs in HCC tissue demonstrated by immunofluorescence. The blue color indicates HCC nucleus, and the green H-CAFs with α-SMA stained. CAFs are circulating the cancer nests in the malignant tissue. HCC: Hepatocellular carcinoma; H-CAF: HCC-associated cancer-associated fibroblasts; α-SMA: α-smooth muscle actin.

α-SMA are highly expressed in tumor stroma, and during transformation from normal fibroblasts to activated CAFs, the expressions of α-SMA, TGF-β and stromal cell-derived factor-1 (SDF-1) are significantly up-regulated with impressive functions exerted. Bone marrow-derived stromal cells like mesenchymal stem cells (MSCs) which share similar functions with CAFs and express α-SMA and a1 chain of type I collagen are also believed to be the source[25]. Tumor microenvironments dynamically regulate the activation of recruiting MSCs, which leads to formation of activated fibroblasts in tumors[26]. MSCs obtain an analogous phenotype with CAFs after co-culture with tumor cells or treatment with tumor conditioned medium *in vitro*, expressing higher levels of α-SMA, FSP, vimentin and SDF-1/CXCL 12[26]. Epithelial mesenchymal transition (EMT) of malignant and normal epithelial cells is a possible mechanism, and E-cadherin could be down-regulated by TGF-β[27]. Vascular endothelial cells resemble CAFs in that they both express CD31 as well as α-SMA and FSP, when induced by TGF-β, and are inferred to partly migrate from vascular basement membrane to tumor stroma and transdifferentiate into CAFs[27]. Transformation of senescent fibroblasts may also be an approach. No evidence of clonal somatic genetic alterations has been detected in CAFs[28]. It is likely that CAFs originate from various cells. During co-culture of breast cancer cells and fibroblasts, CAFs were mostly derived from surrounding normal fibroblasts, with a small portion from vascular smooth muscle cells and pericytes[29].

CAFS AND CARCINOGENESIS

A number of studies have reported crucial roles of CAFs in providing cancer cells with proliferative and survival propensities favoring tumorigenesis[30]. CAFs play important roles in tumorigenesis, and it is possible that before canceration of epithelial cells, fibroblasts in stroma are activated to transform into CAFs, which secrete tumor-promoting factors[30]. Fibroblasts in the skin of individuals susceptible to breast cancer express abnormal phenotypes which reduce their requirements for culture *in vitro* compared to normal fibroblasts, and enhance their proliferative ability; and skin fibroblasts segregated from patients with melanoma, retinoblastoma, Wilms carcinoma and multiple colon polyps also demonstrate potent proliferation potential, indicating that fibroblasts *in vivo* may promote tumorigenesis[31]. Olumi *et al*[32] found that when eternal but not tumorigenic prostate epithelial cells were inoculated into nude mice with/without fibroblasts originating from normal prostate tissue, they proliferated faintly without oncogenic ability; but when they were co-transplanted with CAFs, a tumor was gradually formed. CAFs stimulate malignant cell proliferation by providing various types of growth factors and cytokines, such

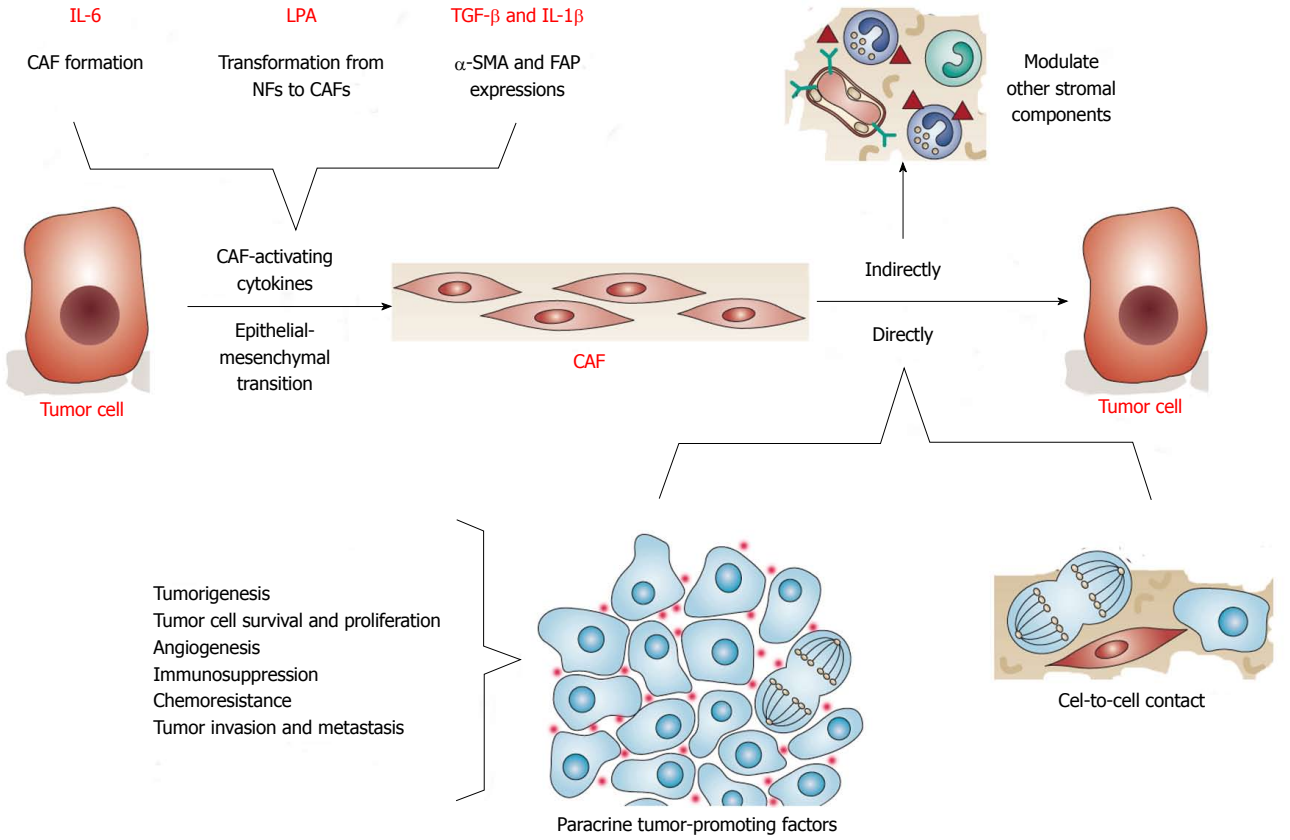

Figure 2 The interplay between cancer-associated fibroblasts and tumor cells. Tumor cells activate CAFs by secreting various cytokines and epithelial-mesenchymal transition; CAFs exert their tumor-promoting function through direct (paracrine soluble factors and cell-to-cell contact) and indirect (modulating other stromal components) approaches (Elements adapted from Mueller *et al*[110]). CAF: Cancer-associated fibroblast; LPA: Lysophostatidic acid; TGF-β: Transforming growth factor-β; NF: Normal fibroblast; α-SMA: α-smooth muscle actin; IL: Interleukin.

as hepatocyte growth factor (HGF), epidermal growth factor (EGF) family members, fibroblast growth factor (FGF), Wnt families, SDF-1 (CXCL12), fork-head box F1 (FoxF1) and IL-6[33-35]. Interestingly, HGF stimulates the growth of some tumor cell lines, but inhibits the growth of some other tumor cell lines, and does not promote proliferation all in a dose-dependent manner in diverse cell lines, which could possibly be explained by the fact that HGF activates various signaling pathways including PI3K, $pp60^{c\text{-}src}$, phospholipase C_γ, c-Met and Ras-ERK, and that cells harboring different dominant pathways react differently to HGF with differences existing in the expression of receptors which are responsive to HGF in cells of diverse origins[36]. CAFs are reported to be able to turn on complementary metabolic pathways to buffer and recycle products of anaerobic metabolism, modifying local severe conditions and facilitating the maintenance and enhancement of cancer cell survival in unfavorable environments such as necrosis with low pH and hypoxia, since they prefer to undergo anaerobic glycolysis even in the presence of oxygen during cancer cell proliferation[37]. Our study further supports that CAFs markedly promote malignant cell growth in HCC, and that CAFs-secreted HGF plays a vital role in cancer cell proliferation[38].

CAFS AND TUMOR PROGRESSION

Tumor progression is now recognized as the product of evolving crosstalk between different cell types within tumors and in the tumor-surrounding stroma[39]. During tumor progression, stroma contributes a suitable microenvironment facilitating tumor invasion, during which CAFs communicate with malignant cells actively and closely. Increasing evidence has revealed that CAFs facilitate tumor growth and progression by synthesizing and releasing diverse soluble molecules in a context-dependent manner including growth factors, chemokines, proteinases and ECM components, among which SDF-1, EGF, HGF and insulin-like growth factor (IGF) are considered typical[9,40-42]. In particular, HGF plays an important role not only in tumor growth, but also in tumor transformation promotion, facilitating tumor metastasis *via* classic HGF-cMet signaling pathway activation. It has been proved that blocking HGF secretion by fibroblasts reduces metastasis rates[43]. CAF-secreted prostaglandin E2 (PGE2) has also been proved to promote tumor growth in head and neck tumors[44]. Decreased Pten expression in CAFs in tumor stroma can promote tumor cell growth[20]. In HCC, hepatocellular CAFs have also been proved to pro-

mote malignant cell proliferation *in vitro*[16].

Non-invasive tumor gains invasive ability after co-inoculated with CAFs in nude mice, and CAFs in breast cancer were reported to stimulate angiogenesis by releasing SDF1α+β[45]. After low-invasive cancer cells were inoculated into the skin of nude mice with corresponding CAFs or normal fibroblasts from patients with inclination of metastasis, those co-inoculated with CAFs had significantly more potent proliferative and invasive abilities, with richer surrounding collagen. This was due to the fact that CAFs can recruit bone marrow-derived endothelial progenitor cells into the stroma, facilitating angiogenesis, remodel the ECM by enhancing integrin signaling through matrix crosslinking, and promoting tumor growth and metastasis by secreting abundant soluble tumor-promoting factors such as SDF-1/CXCL12, which are also highly expressed when cultured alone, further demonstrating that CAFs take an active part in tumor progression[46-49]. In a melanoma-liver-metastasis model, the formation of metastases was found to be closely associated with activation of HSCs, also suggesting the tight relationship between CAFs and metastasis[50]. CAFs can also increase the invasive ability of esophageal cancer cells *via* secretion of HGF[51].

CAFs can also interfere with tumor growth, invasion and metastasis by secreting abundant angiogenic factors and matrix metalloproteinases (MMPs). After fibroblasts are co-cultured with invasive melanoma, they secrete significantly more cytokines including IL-1β, IL-8 and IL-6, and inhibiting the synthesis or secretion of those factors attenuates the invasive ability of malignant cells, suggesting the key role of those molecules[52]. The interaction between cytokines and proteinases secreted by CAFs and ECM components is also a vital progression and metastasis mediator.

MMPs can not only promote tumor invasion and migration by directly acting on cancer cells, but also participate in reconstruction of ECM after degrading it, thus facilitating tumor cells to penetrate the extracellular barrier, infiltrate and escape. For instance, MMP3 can directly act on protein E-cadherin in the extracellular region of tumor cells, decomposing the formed compound with the function of cell adhesion, and facilitating EMT and invasion of tumor cells[53]; MMP1 can activate protease-activated receptor 1 (PAR1) by degrading its extracellular region, which promote invasion and metastasis of tumor cells *via* PAR1-dependent Ca^{2+} signaling[54].

IL-6, an inflammatory factor mediating acute inflammation, is now believed to be another key cytokine regulating tumor progression. After the IL-6/STAT3 pathway is activated, malignant cells proliferate much faster and their anti-apoptosis ability increases significantly[55]. In the liver, IL-6 can directly promote the regeneration of hepatic cells, and inhibit apoptosis mediated by Fas[56].

CAFS IN PRIMARY TUMORS *VS* IN METASTASES

Abundant evidence supports the pro-metastasis role of CAFs in primary tumors, however, there is limited data on CAFs in the corresponding lymph nodes and distant metastases, although accumulating evidence shows crucial roles for CAFs in metastases. Stromal signals resembling those of a distant organ can select for cancer cells that are primed for metastasis in that organ[57]. Metastatic cancer cells can bring their own soil, including CAFs, from the primary tumor to the metastases[58]. MSCs incorporated into the stroma of the primary tumor and metastases both expressed α-SMA and PDGFR-β as CAF markers[59]. Stromal reactions in metastases, probably containing metastasis-associated fibroblasts (MAFs), have been described as reactive and fibrotic tissue with enhanced deposition of vitronectin and fibronectin, desmoplasia, nodal fibrosis and hyaline stroma[60,61]. Kaplan *et al*[62] showed that tumor-secreted factors promote metastatic spread into specific distant organs. Within days after primary tumor implantation, localized deposition of extra domain (ED)-A or ED-B spliced forms of fibronectin occurs due to resident fibroblasts within target organs that are conventional sites of metastasis, corresponding to the particular primary tumor. It may be that TGF-β responsiveness is equally important at the metastatic site as in the primary tumor, as cellular forms of fibronectin and lysyl oxidase (LOX) are well-known TGF-β target genes[63-65]. Chaiwun *et al*[66] reported that loss of Glutathione S-transferase Pi (GSTPi) expression was observed in breast cancer cells in paired cases of both the primary invasive breast cancer and the corresponding axillary lymph node metastases compared with benign breast epithelial cells, and that a significant association exists between GSTPi-, vimentin- and α-SMA-positive fibroblasts in the tumor microenvironment at both sites. CAF conversion from ostensibly normal cells is mediated by various factors including TGF-β[67,68], Act A[69], MMP7[70], PDGF[71], prograstin[72], sonic hedgehog[73], and YAP[74], while factors implicated in MAF corruption are osteopontin[75], discoidin domain receptor 2 deficiency[76], IQGAP-1 deficiency[77], exosomal microRNA (miR)-494, miR-542-3p[78], miR-31, miR-214, miR-155[79], Met exosomes[80], and TGF-β exosomes[81]. In the diverse steps of the metastatic cascade including invasion, ectopic survival, stimulation of angiogenesis, adhesion at the metastatic niche and macroscopic metastasis formation[82], CAFs continuously transmit profitable signals interpreted as various factors including CCL2/FGF19[83], CD81 exosomes[84], EGF, IGF-1[85], S100A4[86], stanniocalcin (STC)[87], track formation[88,89], type I collagen alignment[90], and direct cell-cell contacts[91], while factors implicated in MAFs-induced metastasis include CXCL10[92], ED-A/ED-B fibronectin[62], IL-11[65], and periostin[93], with LOX[63,94], neuregulin 1[95], SDF-1[77,96], scatter factor (SF)/HGF[77,97,98], and tenascin-C[97,99] as the shared factors between CAFs and MAFs. Based on CAF-specific signatures present in exosomes, we may detect and monitor CAF reactions, and probably MAF formation allowing optimized prognostic values[82]. The differential gene expression patterns between normal colonic fibroblasts, CAFs from primary tumors and CAFs from hepatic metastasis could be useful

for predicting relapse in primary tumors[100].

CAFS AND TUMOR EMT

EMT is physiologically important during the development of multicellular animal embryos and the formation of tissues and organs, and is commonly found in many pathological processes including wound healing, inflammation and invasion. It is considered to be the most important and common process during tumor metastasis, and has become a hot topic in recent years[101]. Epithelial cells undergo EMT when the cells which are closely arranged with weak deformability are gradually transformed into stromal cells which are loosely arranged with strong mobility by triggering and inducing factors[102]. In tumors, EMT occurs in malignant cells when non- or weakly-invasive tumor cells with epithelial phenotype gradually change into cells with strong invasive ability, induced by external factors[103]. Tumor progression is a multi-step process, and EMT is a key link. CAFs or CAF-precursor cells can significantly promote EMT of malignant cells[104]. The major cellular biological behavior change of tumor cells undergoing EMT is enhanced invasive and metastatic abilities[105].

The most important iconic change during tumor cell EMT is the down-regulation of E-cadherin or E-cadherin/β-catenin compound expression, which are important inter-cellular adhesion molecules, and negatively correlate with tumor invasive and metastatic abilities[106]. Reduced expression of E-cadherin would attenuate cellular adhesive ability, promoting tumor migration[107]. However, during EMT, the expression of proteins with stromal phenotypes including fibronectin, N-cadherin, vimentin and α-SMA recurs or increases, and is closely associated with tumor invasive ability[108]. EMT is also accompanied by the increased expression of relative transcription factors including Snail, Slug and Twist[109]. During tumor invasion, tumor cells always lose their epithelial characteristics, and present stromal cell phenotypes, which resembles the EMT during embryo development[103].

Increasing evidence has revealed that EMT of tumor cells is based on the interaction between malignant cells and tumor stroma, during which CAFs play vital roles, by secreting a series of soluble mediators or functioning through the direct cell-to-cell approach[54]. EMT promotes invasion of tumor cells, and it is regulated by various exogenous stimulating factors including the MMP, integrin, and TGF-β factors, which further activate the downstream tyrosine kinase-dependent pathway after binding their corresponding receptors[110]. According to in-depth research, most of the stimulating signals were revealed to be secreted by CAFs, indicating the key role of CAFs during tumor EMT, and CAFs induced EMT of tumor cells by providing them with pre-migration stimulation, generating greater invasive and metastatic abilities[111,112]. During the research in prostate cancer, CAFs were found to induce EMT and stem cell marker expression in cancer cells, degrade ECM, and promote invasion and metastasis by secreting MMP2 and IL-6[15]. CAFs were also found to be closely associated with tumor cell EMT in oral and tongue cancers[10,113]. CAF-precursor cells such as hepatocellular and pancreatic stellate cells can also induce EMT[23]. The significant role of TGF-β released by CAFs during tumor cell EMT was specifically revealed[114], and it has bidirectional functions: in normal tissues, TGF-β can inhibit cell proliferation and promote apoptosis, attenuating carcinogenesis[101]; while during tumor progression after the tumor is formed, TGF-β becomes the ally of tumor, inducing EMT and promoting invasion and metastasis[114]. The conditioned medium of cultured CAFs isolated from invasive breast cancer tissues (CAF-CM) can transform breast cancer cell lines into more aggressive phenotypes by promoting EMT mainly induced by CAF-paracrine TGF-β1 with the TGF-β/Smad signaling pathway activated, and enhancing cell-extracellular matrix adhesion, migration and invasion in breast cancer cells. The EMT phenotype induced could be reversed by blocking TGF-β1 signaling[115]. TGF-β1 does not stimulate CAF with a more divergent expression pattern (CAF-D) migration, but enhances invasion and expression of EMT markers in malignant keratinocytes. Inhibiting TGF-β1 in three-dimensional cultures containing CAF-D impairs keratinocyte invasion, suggesting TGF-β1-induced EMT mediates CAF-D-induced carcinoma cell invasion[68]. Research into other tumors also showed that tumor EMT mediated by TGF-β is usually accompanied by activation of the Smad, PI3K/AKT, and P38 MAPK signaling pathways, the first of which may be major[116]. Other CAFs-secreted growth factors, inflammatory factors and chemokines including HGF, FGF, IGF, tumor necrosis factor (TNF)-α, IL-1β, MCP-1 (CCL2), Rantes (CCL5), MCP-3 (CCL7), endothelin-1 (ET-1) and the integrin family members have also been proved to promote EMT[113,117-119]. Our previous study found that CAFs induce EMT in HCC cells mainly through release of HGF and IL-6, and increase the invasive and migratory properties of the cancer cells[38]. Recently, our ongoing research revealed that during HCC progression, EMT could be induced by CAFs mainly through the transglutaminase 2 (TG2) mediated IL-6/STAT3 signaling pathway.

In research into Lewis sarcoma metastasis, an interesting phenomenon called packaged metastasis was revealed, and tumor cells were found to metastasis alone with its "soil", mainly consisting of CAFs; after removing CAFs, the lung metastasis rate decreases significantly[58].

Ectopic miR-205 over-expression in cancer cells can counteract CAF-induced EMT, thus impairing enhancement of cell invasion, acquisition of stem cell traits, tumorigenicity, and metastatic dissemination. The evidence that miR-205 replacement in cancer cells is able not only to prevent, but also to revert the oxidative/pro-inflammatory axis leading to EMT induced by CAFs sets the rationale for developing miRNA-based approaches to prevent and treat metastatic disease[120].

CAFS AND FEATURES OF TUMOR STEM CELLS AND CHEMO-RESISTANCE OF TUMOR CELLS

Research has shown that after undergoing EMT, malignant cells become more resistant to chemotherapy, and those expressing surface molecules of stem cells increase, suggesting a close link between CAFs-induced EMT, tumor stem cells, and chemo-resistance of tumor cells[42,121-124]. EMT can trigger the transformation of malignant cells to tumor stem cells. In breast cancer research, $CD44^{+}CD24^{-}$ cancer stem cells exhibit activation of the TGF-β signaling pathway, and blocking this pathway results in a decrease in stem cells and a process contrary to EMT, namely mesenchymal-epithelial transition (MET)[121,122]. CAFs are primarily resistant to chemotherapy due to a small proportion of proliferating cells in contrast to malignant cells, which makes CAFs a potential source of tumor progression[125]. CAFs have been proved to promote chemo-resistance of malignant cells in many tumors. Witta *et al*[42] believe that tumors with stromal phenotypes are more chemo-resistant and share more characteristics with tumor stem cells. After culture in conditioned medium of pancreatic CAFs, cancer cells had greater proliferative, invasive and metastatic abilities, forming a mass, and greater resistance, decreasing the rate of apoptosis induced by chemotherapy by up to 76%. Loeffler *et al*[126] reported that elimination of CAFs expressing FAP by a DNA vaccine could significantly enhance the antitumor effect of Doxorubicin, inhibit tumor growth and metastasis, and prolong survival time. CAFs can even mediate tumor resistance to anti-VEGF treatment through PDGF-C secretion[127].

CAFS AND IMMUNITY

Cancer patients exhibit a generalized immunosuppressive status, and there is substantial evidence that stromal activation can foster tumor growth and progression by modulating immunopathogenesis[39]. The role of the tumor environment has been largely studied as a dynamic system orchestrated mainly by inflammatory cells. There is substantial evidence supporting that it is not inflammation *per se*, but rather the inflammatory "context" that determines the ability of pro-inflammatory factors to facilitate or prevent tumor growth[128]. CAFs can stimulate angiogenesis in tumor stroma to provide malignant cells with more nutrients by releasing VEGF and PDGF[129]; attract immune cells including macrophages and neutrophils to induce inflammation by secreting IL-1, IL-6, IL-8 and TNF-α[4]; and participant in reconstruction of the tumor microenvironment by regulating inflammatory cells such as lymphocytes by secreting uPA and MMPs to mediate tumor progression[130]. They can induce inflammation in tumors by secreting chemokines including CXCL1, CXCL2, CXCL5 and IL-6 which activate cancer-associated macrophages to promote tumor invasion and metastasis[4]. Immune invasion occurring among malignant cells is considered to be one of the important causes of poor prognosis. Immune cells ought to be the barrier killing tumor cells by the function of immune surveillance. However, in many solid tumors, immune cells could be "educated" by malignant cells to be the accomplice during tumor progression[131]. CAFs which can produce significant levels of suppressive mediators have been proved to be involved in immunosuppression and tumor immune privilege and escape through functioning on immune cells in the tumor microenvironment[132], and are found to recruit Th17 cells and up-regulate IL-17 expression *via* RANTES and MCP-1 secretion, producing a pro-inflammatory cytokine milieu, providing cell-to-cell contact engagement, and participating in cancer immunosuppression in melanoma, breast cancer, and colon carcinoma[133]. CAFs separated from non-small cell lung cancer tissues have reciprocal interactions with T cells, and can induce and up-regulate the expression of IL-17A and INF-γ in tumor-associated T (TAT) cells *via* IL-6, and when anti-IL-6 is supplied, the regulatory function is weakened[134]. Subpopulations of CAFs can highly express B7H1 (PD-L1) and B&DC (PD-L2), which can induce immune tolerance and be up-regulated by INF-γ; when TATs are co-cultured with CAFs, their function is suppressed, and after anti-B7H1 is added to block the pathway, TATs regain their function completely, suggesting that CAFs can regulate TAT function *via* the PD-L1/PL-1 axis[132]. In particular, natural killer (NK) cells can be potently regulated by soluble factors derived from CAFs under inflammatory conditions. In breast cancer, CAFs are reported to be unable to stimulate effective antitumor NK cell responses and to suppress NK cell cytotoxicity[135]. In metastatic melanoma, CAFs can trigger NK cell dysfunction by secreting PGE2, which is characterized by suppressed expression of NKp44 and NKp30, also suggesting that CAFs can prevent malignant cells from being eliminated by immune cells, facilitating tumor cell growth and metastasis; interestingly, NK cells can also trigger a paradoxical suppressive loop by enhancing the production of PGE2 by CAFs[136]. Our previous study also indicated that hepatocellular CAFs could trigger NK cell dysfunction and suppress their activation by secreting PGE2 and indoleamine-2,3-dioxygenase, creating an unresponsive condition in tumors. This condition is characterized by low expression of cytotoxic molecules and surface markers for cell activation, impaired production of cytokines, and decreased cytotoxicity against malignant cells[137]. CAFs are frequently present in the stroma of colorectal carcinoma tissues[138], and we also found that colorectal CAFs exhibit activated phenotypes and sharply suppress NK cell functions with secretion of PGE2, indicating a novel mechanism linking the pro-inflammatory response to immune tolerance within the tumor milieu[139]. In contrast and interestingly, NK cells also play an antifibrotic role *via* an inhibitory effect on myofibroblasts, which secrete TGF-β and PDGF to enhance tumor growth and progression[140], by inducing apoptosis and *via* the production of INF-γ, and downregulation of NK cells may facilitate liver

fibrosis[141]. It is the general view that the tumor microenvironment induces tolerance[142], and this is worthy of further investigation to identify the roles of CAFs in immune editing. Manipulating the expression of and signaling through these molecules may open new rational avenues for developing novel immune-based therapies to enhance antitumor immunity in human cancer.

CAFS AND PROGNOSIS

CAFs are closely associated with prognosis. It was validated in the VICTOR trial that the proportion of intratumor stroma can serve as a significant prognosticator for stage Ⅱ and Ⅲ colon cancer patients[143]. The overall and disease-free survival periods (OS and DFS) were significantly lower in the stroma-high group [OS, hazard ratio (HR) = 1.96; DFS, HR = 2.15]. The 5-year OS was 69.0% *vs* 83.4% and DFS 58.6% *vs* 77.3% for stroma-high *vs* stroma-low patients with appreciable differences. The protein, FAP, is specifically expressed by CAFs. A retrospective study showed that expression of FAP in tumor stroma was positively correlated with metastatic lymph nodes, metastases and recurrences, and negatively correlated with prognosis[138]. However, research into breast cancer has drawn the opposite conclusion, revealing that high expression of paracancerous FAP was positively correlated with tumor-free survival, and negatively correlated with metastasis, indicating that FAP could be regarded as an independent prognostic marker[144]. It is unknown why diametrically opposite results have been demonstrated in diverse tumors. Research into primary HCC showed that paracancerous activated HSCs were negatively correlated with clinical outcomes, but positively correlated with early metastases, and that HSCs along with Tregs and monocytes could be treated as effective prognostic indicators[25]. The level of IL-6 secreted by CAFs was proved to be correlated with clinical stage of HCC[145]. Our study also suggests the prognostic significance of CAFs in HCC[38]. Our ongoing study revealed that TG2 in CAFs may serve as a novel prognostic biomarker and therapeutic target.

CAFS AND TARGETED THERAPY

Targeted therapy toward CAFs has displayed promising anticancer efficacy, further strengthening the need to study the correlation between CAFs and their hosts[146]. CAFs can regulate carcinogenesis and tumor progression by secreting soluble mediators including cytokines, chemokines, proteinases, collagens and fibronectins. Nowadays, there are inhibitors and antibodies which target cytokines, chemokines and proteinases, with the advantage that CAFs have a relatively stable genome making it nonsusceptible to various drugs, suggesting that CAFs-targeted therapy is theoretically practicable and clinically operable. Anti-cancer therapy targeting CAFs has been actively investigated recently. An antibody against FAP inhibited the growth of colon cancer[147]. A number of drugs targeting receptors on the cellular surface have been applied clinically, and PDGF receptors such as regarofenib, imatinib (Glivec), sorafenib and sunitinib have been used to treat patients with metastatic gastrointestinal stromal tumor (GIST), renal carcinoma, and hepatocellular carcinoma. An MMP inhibitor (MMPI) with better selectivity has also been investigated in clinical trials[148]. A phase I clinical trial[149] on a mono-antibody targeting PD-1 in the treatment of solid tumors has been completed which showed stable and satisfactory efficacy. Research into CAFs will provide a theoretical basis for novel strategies of tumor stroma-targeted early diagnosis, prognosis-prediction and new preventive and therapeutic methods, which are of great potential clinical value. However, further investigations into CAFs are required.

CONCLUSION

The current trend in cancer research is the inclusion of the tumor microenvironment as a major contributor to malignant progression[9]. The importance of the stromal tissue in regulating the physiological processes of the body is undeniable. The reviewed studies emphasize the importance of the cross-talk between stroma and malignant cells (Table 1). Key evidence demonstrates that stromal components may modulate cancer progression. The stromal cells of the carcinogenic lesion, especially CAFs, have long been known to be supportive and responsive, and express critical signals that drive proliferation, angiogenesis, and motility. CAFs are thought to be activated, characterized by the expression of various markers including α-SMA, FAP, FSP, vimentin, fibronectin, *etc*., and may have multiple origins including normal and aged fibroblasts, MSCs, vascular endothelial cells, and importantly, EMT. In most digestive solid tumors, CAFs influence epithelial transformation by direct cell-to-cell contact or by producing paracrine factors that affect both normal epithelia as well as carcinoma cells. The tissue specificity of the stroma-epithelial interaction possibly accounts for the tissue and cell type-specific role of the microenvironment in carcinoma progression. CAFs also play important roles in immunosuppression induction, and may be a significant prognosticator. The recognition of the active role that CAFs play in carcinogenesis not only adds a new level of complexity to cancer biology, but also brings an opportunity for new therapeutic strategies. Targeted therapy towards CAFs has displayed promising anticancer efficacy.

EMT, which can be induced by CAFs, significantly promotes carcinogenesis, tumor invasion, metastasis and chemo-resistance. It is controlled by a network of regulators, and provides an explanation for the association between inflammation and cancer progression. Progress in understanding EMT has been an exercise in appreciating the level of complexity required to change cellular identity. The mechanism of transition highlights the integration of nuclear regulation and network signaling with alterations in the microenvironment to create a moving

Table 1 Roles of cancer-associated fibroblasts in diverse pathological processes and the molecules involved

Function	Soluble molecules involved
Tumor cell growth and proliferation	HGF, EGF family members, FGF, Wnt families, SDF-1 (CXCL-12), FoxF1, IL-6, PDGF
Tumor invasion and metastasis	SDF-1 (CXCL-12), CXCL10, EGF, SF/HGF, IGF, PGE2, MMPs, IL-1β, IL-6, IL-8, IL-11, CCL2/FGF19, CD81 exosomes, S100A4, STC, type I collagen, ED-A/ED-B fibronectin, periostin, LOX, NRG1, TNC, PDGF
Epethelial-mesenchymal transition	MMPs, integrin family members, TGF-β, IL-6, HGF, FGF, IGF, TNF-α, IL-1β, MCP-1 (CCL2), Rantes (CCL5), MCP-3 (CCL7), ET-1
Chemoresistance	PDGF-C, TGF-β
Immunosuppression	VEGF, PDGF, IL-1, IL-6, IL-8, TNF-α, uPA, MMPs, CXCL1, CXCL2, CXCL5, Rantes, MCP-1, PGE2, indoleamine-2, 3-IDO

HGF: Hepatocyte growth factor; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; SDF: Stromal cell-derived factor; FoxF1: Fork-head box F1; IL: Interleukin; PDGF: Platelet derived growth factor; SF: Scatter factor; IGF: Insulin-like growth factor; MMP: Matrix metalloproteinase; TGF: Transforming growth factor; STC: Stanniocalcin; MCP: Monocyte chemoattractant protein; PGE: Prostaglandin E; LOX: Lysyl oxidase; NRG: Neuregulin; TNC: Tenascin-C; TNF: Tumor necrosis factor; ET: Endothelin; uPA: Urokinase-type plasminogen activator; VEGF: Vascular endothelial growth factor; IDO: Indoleamine-2,3-dioxygenase.

cell. Here we have focused on the effects of various stroma-derived paracrine factors on epithelial cells. It is probable that these same factors also have vital effects on other stromal cells, including fibroblasts, endothelial cells and inflammatory cells. The malignant state occurs and is exacerbated by defects in communication pathways which recruit host cells to become vital participants in the heterotypic tissue invasion field. Cross-talk between tumor cells and host cells triggers pro-survival, proliferation and invasion pathways in both the cancer cells and their host. Some of the EMT-associated signal transductions point to potential targets for therapy. A better understanding of the effects of targeting these pathways, however, is required. It is likely that further characterization of these interactions, and the molecular identification of key mediators, will provide novel insights into oncology and indicate new therapeutic options. Pharmacological and biological agents that interfere with signaling between the malignant epithelial cells and the supporting stroma will likely continue to be tested. An important issue for EMT identification to be regarded as having a high prognostic and therapeutic value is to determine the use of specific markers. These should, in theory, be able to recognize a cancer EMT-derived mesenchymal cell from a normal mesenchymal cell.

Recent findings have produced great strides in developing an understanding of the molecular events involved in processes necessary for tumor cell invasion and subsequent metastasis formation. However, it is unclear which signaling pathways should be inhibited in order to most effectively block tumor progression and cause minimal toxicity in normal tissues at the same time. It is also notable that the models described in this review are not mutually exclusive, both in different patients, as well as in different fibroblastic cells in any specific tissue, as fibroblasts are quite heterogeneous. Most of the valuable insights concerning the tissue microenvironment have been derived from co-culture and tissue recombination xenograft experiments, however, these results may not be applicable to the *in vivo* situation due to the fact that not all key environmental factors and cells are considered. The ability to overexpress specific factors or conditionally knock out specific genes *in vivo* in stromal cells will further add to our knowledge of the complex interactions involved in cancer progression. Future developments will include a new class of therapies targeting the extracellular and intracellular mediators, which act at the tumor-host communication interface. Future efforts should further focus on how the events of cell attachment, matrix proteolysis, and cell migration are controlled and integrated, which requires a better understanding of the transcriptional regulations and cell signaling mechanisms that are involved in these events. We have relatively poor knowledge regarding the similarities or differences between CAF function in the primary tumor, pre-metastatic niche and metastasis. Future work should also aim to define the function, identities and molecular profiles of diverse CAF subtypes in tumor biopsies from primary tumors and from metastases pre-, during, and post-treatment. In addition, animal models should be carefully designed to allow early identification and to clarify the role of MAFs.

In conclusion, it is necessary to explore the relationship between CAFs and their hosts. In addition, further understanding of the relevant genetics and epigenetics will be especially vital in order to differentiate CAFs as patient-specific therapeutic targets.

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Enterolithiasis

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Abstract

Enterolithiasis or formation of gastrointestinal concretions is an uncommon medical condition that develops in the setting of intestinal stasis in the presence of the intestinal diverticula, surgical enteroanastomoses, blind pouches, afferent loops, incarcerated hernias, small intestinal tumors, intestinal kinking from intra-abdominal adhesions, and stenosing or stricturing Crohn's disease and intestinal tuberculosis. Enterolithiasis is classified into primary and secondary types. Its prevalence ranges from 0.3% to 10% in selected populations. Proximal primary enteroliths are composed of choleic acid salts and distal enteroliths are calcified. Clinical presentation includes abdominal pains, distention, nausea, and vomiting of occasionally sudden but often fluctuating subacute nature which occurs as a result of the enterolith tumbling through the bowel lumen. Thorough history and physical exam coupled with radiologic imaging helps establish a diagnosis in a patient at risk. Complications include bowel obstruction, direct pressure injury to the intestinal mucosa, intestinal gangrene, intussusceptions, afferent loop syndrome, diverticulitis, iron deficiency anemia, gastrointestinal hemorrhage, and perforation. Mortality of primary enterolithiasis may reach 3% and secondary enterolithiasis 8%. Risk factors include poorly conditioned patients with significant obstruction and delay in diagnosis. Treatment relies on timely recognition of the disease and endoscopic or surgical intervention. With advents in new technology, improved outcome is expected for patients with enterolithiasis.

Key words: Enterolithiasis; Gallstone ileus; Intestinal obstruction; Crohn's disease; Meckel's diverticulum; Diverticulosis; Intestinal tuberculosis

Core tip: We review classic descriptors and latest developments in the enterolithiasis. The article focuses on detailed description of medical epidemiology, classification, pathophysiology, etiology, clinical presentation, differential diagnoses, clinical diagnosis, management, complications, and prognosis of the enterolithiasis. We mention latest trends in endoscopic approach to patients with symptomatic disease. Our paper serves a first comprehensive review of the syndrome for a practicing gastroenterologist.

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INTRODUCTION

Enterolithiasis, or presence of stone concretions in the gastrointestinal tract, is an important but relatively uncommon clinical condition that has recently gained significant attention with advances in the gastrointestinal field. Primary enteroliths arise in areas of intestinal stasis in the setting of diverticular disease, surgical enteroanastomosis, blind pouches, and intestinal stenosis or strictures seen in the infectious or inflammatory bowel diseases. Secondary enteroliths include gallbladder and renal stones that may

migrate into the gastrointestinal tract as a result of fistula formation. Clinical enterolithiasis is largely affected by its etiology, underlying conditions, age, gender and geographic distribution. Presentation is often nonspecific, but typically includes "tumbling" abdominal pain, nausea, and vomiting related to the bowel obstruction, and may potentially lead to the gastrointestinal bleeding and perforation. To date, enterolithiasis was largely reported in single case observations or case series. In this latest review, we discuss history, epidemiology, classification, pathophysiology, etiology, clinical features, diagnosis, treatment, complications, and prognosis of this gastrointestinal disease.

HISTORY

Enterolithiasis was first described by a French physician Chomelin J in 1710 in the medical series of *Historie de l' Academie Royal*[1] as a case of stone formation in a duodenal diverticulum that was discovered during an autopsy. This entity received more attention in the early twentieth century with additional reports of patients presenting with small bowel obstruction due to a lodged stone in the distal ileum. Described by Williams in 1908 and later consolidated in a literature review of existing 15 cases by Edwards[2] in 1930, this syndrome has gained momentum with the rapid development of gastrointestinal and radiology fields. Sjoqvist initially reported on chemical composition of enterolith in 1908[3]. Pfahler *et al*[4] acknowledged the first radiologic diagnosis of alimentary stone in 1915. By the mid of the century, De Witt *et al*[5] reported his experience with enterolithiasis across a wide age populationand further light was shed by additional individual case reports. It has then become readily recognized that calculus formation in the intestine was a result of an acquired anatomic pathology that predisposes to stasis in the intestinal tract and leads to elemental clumping and aggregation. Diverticular disease of the small bowel was a leading etiology, followed by stricturing of the intestine from infectious (tuberculosis) or inflammatory (Crohn's disease) processes. In 1947, Grettve[3] proposed classification of enteroliths into primary and secondary types, with primary enteroliths being formed inside the gastrointestinal tract and secondary enteroliths introduced from outside the bowel. However, with increased attention to this syndrome, Frink[6] reported on only fewer than 25 cases of enteroliths in the literature up to 1952. In their classic 1960 paper, Atwell and Pollock[7] have managed to further organize primary enteroliths into distinctive true and false subcategories having paid particular attention to the analysis of chemical composition of the stones as related to their location in the gastrointestinal tract. The topic of enterolithiasis has continued to expand and by the second decade of the twenty first century, over a hundred cases have been reported, although not critically reviewed in a dedicated manner.

EPIDEMIOLOGY

The reported prevalence of primary and secondary enterolithiasis in selected populations varies widely from 0.3% to 10% and is largely dependent upon clinical presentation, etiology, and underlying risk factors. Intraluminal stone formation of various sizes is more common than anticipated, typically remains underreported in absence of clinical symptoms or due to its diminutive size that permits intermittent passage, and may not be visualized on conventional radiologic imaging in majority of the cases. However, clinically significant enteroliths are more likely to develop in certain medical conditions. Primary enteroliths are classically formed in the areas of stasis within the bowel in the presence of the intestinal diverticula, surgical side-to-side enteroanastomoses, blind pouches (cul-de-sac), afferent loops in the Billroth Ⅱ gastrojejunostomy and Roux-en-Y procedures, incarcerated hernias, small intestinal tumors, intestinal kinking from intra-abdominal adhesions, or proximal to the intestinal strictures encountered in cases of Crohn's disease and intestinal tuberculosis[8-18].

It is difficult to quantify true incidence of the enterolithiasis in the setting of small intestinal diverticular disease. In fact, significant primary enteroliths remain uncommon medical phenomena, with isolated case series or reports in the setting of typical duodenal and jejunal diverticulosis as well as in special cases of Meckel's diverticulum of the ileum. It should be noted that while the described incidence of jejunal diverticulosis varies from 0.06% to 1.3% in older patients[19], diverticulosis-associated enterolithiasis should be even less common. Prevalence of enterolithiasis in patients with Meckel's diverticulum is best studied to date, and is estimated at 3%-10%[20-22]. Fifteen cases have been reported of post-surgical enteroliths forming after hepatojejunostomy, Billroth Ⅱ gastrojejunostomy, Roux-en-Y gastroenterostomy, and Whipple's procedures[10,23]. There are over 80 cases of enterolithiasis reported in association with intestinal tuberculosis, although most were described by Bery in a single study[24,25]. Small early series of Indian tuberculosis patients showed radiopaque enteroliths in 3% of the patients[26]. However, true prevalence of enterolithiasis in intestinal tuberculosis remains unknown, with large cohorts in studies by Prakash and Deka describing only select cases of the enterolithiasis[24,25,27]. By the turn of the 21st century, there have been an estimated 30 cases reported in association with Crohn's disease[28]. Improved surgical techniques, medical management of chronic intestinal conditions, dietary consumption of calcium products, and finally wide spread use of acid suppressive therapy may alter conventional norms of traditional enterolith formation. Therefore, the true incidence and prevalence of primary enterolithiasis remain to be determined. Additional epidemiological and histological studies are necessary to delineate etiological relationships of enterolithiasis in selected patient popula-

tions. Most cases of enterolithiasis are discovered in symptomatic patients, who present with abdominal pain or small bowel obstruction; thus, the prevalence of asymptomatic enteroliths is still largely unknown. A study by Pantongrag-Brown *et al*[29] in 1996 evaluated 84 patients with Meckel's diverticulum and described 8 (10% prevalence) cases of enteroliths at the time of surgery, a much higher rate than what had been previously reported. Alternatively, a review of 1476 adult patients found to have Meckel's diverticulum during surgery in the Mayo clinic found that enteroliths were seen in 6% of symptomatic and 0.7% of asymptomatic patients at the time of surgery[30].

Gallstone ileus remains the most common form of secondary enterolithiasis. This condition arises in estimated 0.3%-0.5% of general cholelithiasis[31,32]. It accounts for approximately 1%-4% of all cases of mechanical bowel obstruction, while significantly increasing to 25% in geriatric population[31,32-39]. Over-diagnosis of intestinal obstruction due to migrated gallstone has been reported in the literature, largely due to under-recognition of primary "pseudogallstone" enterolithiasis. There are two case reports in the literature of secondary enterolithiasis due to renal stones from an underlying fistulizing disease[40,41].

Gender and age remain important epidemiologic factors in enterolithiasis. As a rule, gender predilection seems to be largely dependent upon the etiology of the stone formation and typically parallels gender predisposition of the underlying condition. Enterolithiasis of Meckel's diverticulosis shows a 3:1 male to female ratio, at least partially due to the 2:1 male to female ratio classically seen in this condition[29]. In contrast, enterolithiasis associated with intestinal tuberculosis and gallstone ileus have shown a female preponderance, likely due to the greater incidence of these diseases in females[11,34]. A review of the 34 cases of enterolith ileus in jejunal diverticulosis reported nearly equal gender distribution[42]. Gallstone ileus clearly has female and geriatric predilection[38].

Enterolithiasis remains a disease of the adulthood, but differs in time of presentation in various congenital and acquired conditions. Thus, Pantongrag-Brown *et al*[29] and Steenvoorde *et al*[42] reported mean age of 45 and 70 years in Meckel's diverticulum and jejunal diverticular disease related enterolithiasis, respectively. In earlier reports, proximal small bowel enterolithiasis was seen in older adults with average age of 65 years and distal small bowel stone formation was seen in a younger group of patients with average age of 50 years[7]. A series of Crohn's patients showed a mean duration of symptoms 15.7 years before presenting with enterolith, likely reflecting the increasing incidence of stenosis and adenocarcinoma with time[28,43]. In Nakao's analysis of 176 cases of gallstone ileus, the affected patients ranged from 24 to 91 years of age[33] while Ayantunde *et al*[34] reported a mean age of 77 years in patients with this condition.

CLASSIFICATION

Categorization of enteroliths into primary and secondary types helped organize classification system that we use today. Primary enteroliths are formed within the gastrointestinal tract and can be further subdivided into the "true" and "false" subtypes. True primary enteroliths are made of substances found in chyme under normal alimentary conditions, may occasionally have a central "fruit pit", and are subdivided into the choleic acid and calcium (calcium phosphate, calcium oxalate, and calcium carbonate) stones. False primary enteroliths are formed from insoluble foreign substances in the bowel and are divided into three types: agglutination of a large amount of indigestible materials (bezoars), precipitation of substances in the intestinal tract that become insoluble because of resorption of their solvents (varnish stones in varnish drinkers), and concentration of water suspended insoluble salts (chalk, lime, barium sulfate). Mixed concretions may be seen in cases of eventual external calcification of false enteroliths in the distal small bowel[7]. Secondary type enteroliths are stones that are formed in the organs outside of the proper gastrointestinal tract and then migrate into the bowel causing obstruction, with the most common type being gallstones[14,28].

ANATOMY AND PATHOPHYSIOLOGY

Anatomy of the intestinal tract plays an integral role in enterolith formation. Luminal pH and microenvironment specific to each segment of the gut, coupled with development of diverticular disease, altered endoluminal propagation and peristaltic functionality are important factors in developing conditions necessary for stone formation. The small intestine is 3 to 7 meters long and is divided into three regions: duodenum, jejunum, and ileum. Physiologic enteral motor and sensory functions play an important role in the proper aboral movement of chyme and indigestible residues along the small intestine. Integrity of the intestinal tract allows for unobstructed and proper peristalsis and is essential in maintaining continuous migration of the chyme and the endogenous secretions. Importantly, endoluminal microenvironment plays a crucial role in its digestive properties. Under physiologic conditions, established small intestinal pH variation is region specific, starting at about pH of 6 in the duodenum and progressively increasing to about pH 7.4 in the terminal ileum[44].

True primary enteroliths are formed from chemical elements already present in the bowel in the anatomically compromised areas of stasis and their composition may indeed vary by location. Choleic acid enteroliths that require lower pH are typically found in the proximal small intestine, largely affected by significant diverticular disease, strictures or stenosis. On the other hand, calcium phosphate, calcium oxalate, and calcium carbonate primary enteroliths are found in the distal small bowel. These salts are soluble in water and acidic environments, requiring an alkaline pH to precipitate and thus are most often formed in the terminal ileum[15,43,45]. Of interest, while distally recovered enteroliths may typically contain a small percentage of choleic acid, proximal stones are entirely

calcium free[7]. Under normal physiologic conditions, microlithiasis that may be formed in various gastrointestinal disorders is ultimately cleared by effective propagation of the endoluminal contents without significant delay in segments of the small bowel. On the other hand, prolonged and significant intestinal stasis creates favorable endoluminal conditions for particulate aggregation leading to enterolith formation. Similar to the proximal enteric region, stasis in the distal small bowel may occur in a variety of clinical conditions, including stricturing or stenotic Crohn's disease, post-surgical anatomical alterations, post-radiation enteritis, gastrointestinal tuberculosis, and congenital or acquired ileo-jejunal diverticular disease.

While also at risk of presenting at the site of underlying enteropathy, false enteroliths have different chemical composition. Some enteroliths are made up of large amounts of indigestible ingested material, such as hair, occurring in trichotillomania or trichophagia. Others may be composed of varnish in cases of varnish drinkers, or barium sulfate, chalk, lime, milk, magnesium, and aluminum antacids. Stones can also be formed of fecal matter[14,46].

The chemical composition of secondary enteroliths depends on the etiology of the stone. Gallstones are primarily made of bile acids, lecithin and other phospholipids, and cholesterol[47]. Anatomic proximity of the gallbladder to the alimentary tract increases the risk of fistula formation and gallstone extravasation into the gut in various entero-vesicular disease states. In gallstone ileus, gallstones migrate through fistulas and become lodged in the gastrointestinal tract with the most common site of obstruction in the ileum (60%), followed by the jejunum (15%), stomach (15%), and colon (5%)[48]. Similarly, but less commonly observed, close proximity of the renal pelvis to the second part of the duodenum may predispose patients to renal stone erosion into the small intestinal tract[40,41].

CHEMICAL COMPOSITION AND STRUCTURE

Chemical composition of the primary enteroliths varies by the site of the stone formation, length of its migration in the intestine and finally, the location of the site of its impaction. Concentration of choleic acid in the enterolith is inversely related to the distance from the proximal small bowel to the ileocecal valve, with highest saturations (52%-84%) found in the duodenum and jejunum, while effectively decreasing to single digits distally[7]. Free fatty acids and neutral fats effectively follow the pattern and their composition ranges from 9.6% proximally to 0.6% distally. On the other hand, calcium salt composition of the enteroliths increases proportionally with its location in the distal small bowel. Whereas calcium containing enteroliths are not seen in the proximal intestine, its concentration nears 85% in the distal part of the organ. Various calcium salts are present, including calcium oxalate, calcium phosphate, calcium carbonate, and calcium sulfate in order of decreasing incidence[7]. Calcium dihydrate and uric acid stones were also reported[49]. However, it should be noted that chemical composition of an enterolith may not follow general rule of site-related chemical composition: in cases of acute migration of proximal stone with resultant bowel obstruction or in special case of Meckel's diverticulum with ectopic gastric mucosa that may predispose to the in-situ choleic stone formation.

Enteroliths may vary in size and number, ranging from few millimeters to 10 cm in greatest dimension. They may appear in a single stone, in a cluster of concretions, or in a disseminated pan-intestinal pattern. Enteroliths may range in number from several stones to nearly a hundred[11], largely depending on underlying pathology. While classically round, oval, discoid, or faceted, they may appear as triangular or rectangular, and a small number of stones may be needle-shaped or a phenomena likely related to the original "nucleus" of the stone, its anatomical origin, and route of migration[11,25]. Size and chemical composition of an enterolith may affect is weight, with larger egg-shaped concretions potentially reaching under 100 g[50].

ETIOLOGY

Primary enterolithiasis is typically multifactorial with numerous anatomical and microenvironmental factors predisposing to development of clinically significant concretions. Variations in structural integrity of the intestine may be seen in the diverticular disease (congenital and acquired)[8,9]; surgical entero-anastomoses[14], afferent or Roux loops[10], blind pouches[13]; stricturing or stenosing disease of the bowel seen in infectious and inflammatory conditions (tuberculosis, Crohn's disease)[11,12]; radiation or eosinophillic enteritis[15,51]; mucosal diaphragmatic disease[52]; intestinal duplication[53,15]; fistula[54]; malignancy[55]; and finally in apparent kinking of the gut that is found in patients with intra-abdominal adhesions, external compressions, or incarcerated hernias[15-18]. Other conditions associated with increased risk of enterolith production include intestinal aganglionosis, intestinal amoebiasis, and ischemic enteritis[15]. Resultant stasis and impedance of the normal intestinal flow may promote microbiome-accelerated precipitation of insoluble chemical salts and effectively lead to the stone formation. Prolonged dietary consumption of a large calcium load has been proposed as a possible contributing factor in the distal ileal stone formation, but still remains a hypothesis[56]. Exogenous particle ingestion is a risk factor for development of false primary enteroliths. Finally, enterobiliary and enterourinary pathology may place a patient at a higher risk of developing secondary enteroliths that may result in gallstone (common) or renal stone (rare) ileus[40,57].

Diverticular disease of the intestine clearly predisposes to enteric stone formation. While duodenal diverticulosis occurs in up to 22% of the adult population[58], ileo-jejunal diverticular disease is quite rare with an estimated incidence of less than 5%[59]. Jejunal diverticulosis

accounts for over 80% of the non-duodenal disease[19]. Non-synchronous alimentary peristalsis, mucosal valve flap at the diverticular margin, pre-existing intestinal motility disorders, lack of muscular wall component, and an apparent area of "ready to catch" reservoir predispose to effective chyme stasis, bacterial deconjugation of soluble bile acids, and finally de-novo enterolithiasis[29,42,60-63]. True and false enteroliths may form, the latter potentially from a significant stagnation of food particles and debris accumulation within a diverticulum. Meckel's diverticulum is the most common congenital abnormality of the gut occurring in 2% of the patients. Wide-neck Meckel's diverticula represent a special subtype of the small intestinal diverticular disease with preservation of all bowel wall layers, maintenance of inherent peristalsis, and frequent presence of an ectopic tissue all of which make enterolith formation more difficult. Decreased pH in gastric mucosa lined diverticulum may prohibit otherwise potential calcium salt precipitation in the ileum. Delayed diagnosis of Meckel's diverticulum, small intestinal tissue lining of the pouch, alkaline environment, neck edema or inflammation, and focal nidus formation are potential risk factors for development of the stones[22,29,64]. Typical calcification of the stones seen in the Meckel's diverticulum follows a periphery-to-center progression[29].

Alterations in normal anatomy seen in post-surgical patients play an important role in enterolith formation. Side-to-side anastomosis and end-to-side anastomoses with circular muscular fiber division and alteration in peristalsis place patients at a higher risk of developing enteral luminal stasis and stone aggregation[14]. Blind pouch of various lengths may lead to changes in intestinal microenvironment, create a segment of reversed peristalsis, and predispose to eventual bacterial overgrowth that results in accelerated enterolith formation described above[65]. Similar hypomotility is seen in the Roux-en-Y anastomosis or Billroth Ⅱ gastrectomy with disconnection from the main pacemaker function of the duodenum or associated vagal denervation. Post-surgical strictures may lead to stasis as well. Chemical composition of post-surgical enteroliths reflects their position in the gut, with preponderance of choleic acid stones in the proximal relatively acidic afferent limbs and calcium stones in the distal relatively alkaline efferent limbs. In all mechanisms, bowel stasis promotes bacterial overgrowth, which can result in deconjugation of bile salts, causing soluble choleic acid to become insoluble deoxycholic acid and precipitate enteroliths[66]. Afferent limbs are more likely to be acidic, given their connection with the stomach, thus enteroliths formed in this area are most often choleic acid stones[23,66,67].

Intestinal tuberculosis is a rare complication seen in 2% of the patients with pulmonary tuberculosis. While consistently decreasing in the developed countries, its incidence remains high in many parts of the world, keeping it as an important etiology in enterolith formation that was recognized back in the early 20th century. Chronic or subacute intentional obstruction in absence of effective medical or surgical therapy may be present. In fact, up to 60% of the patients with tuberculous enteritis may develop a significant obstruction[68], which may eventually lead to a stone formation or be a direct result of a concretion that was already formed. Low dietary intake of calcium and high phytate containing foods in poor socioeconomic populations may offset the overall incidence of distal small bowel enterolithiasis in patients with intestinal tuberculosis[25].

Stricturing or stenosing Crohn's disease is more common in the developed countries. The etiology of alimentary track stone formation in chronic active Crohn's disease of the small intestine is in fact similar to multiple stricture forming tuberculous enteritis. Mean duration of the disease symptoms to the enterolith formation is estimated at 15 years[28], largely a result of progressive luminal narrowing. Such enteroliths are most often found within the areas of aneurismal, saccular, or dilated parts of the intestine[43,45]. They may be single or multiple, a concept largely related to the number of stenotic or structuring areas of the intestine, each predisposing to stone formation[12]. Common affinity of the Crohn's disease to the neo-terminal ileum makes calcified enterolith formation there most common[43,52]. Areas of entero-enteric or entero-colic anastomosis in Crohn's disease, with or without disease activity, are risk factors for developing alimentary concretions. Incidentally, enterolith formation may lead to early diagnosis of a location of significant stricture or neoplasm in patients with small intestinal Crohn's disease.

False primary enteroliths are formed from indigestible materials, including trichobezoars that may grow by accumulation of indigestible materials and food rich in cellulose. Intestinal migration of these concretions is rare. Excessive varnish consumption may lead to reabsorption of premixed solvents and significant chemical precipitation in water and digestive juices resulting in varnish stone formation. Effective water resorption from oral solutions of barium sulfate, chalk, lime, milk, magnesium, and aluminum containing antacids may trigger apparent insoluble salt precipitate in the gut. Finally, fecaliths may form in the elderly, psychiatric, bedridden, or narcotic dependent patients with history of chronic constipation[14,46]. Gallstone ileus represents classic type of secondary enterolithiasis. A rare cause of estimated 1%-3% of mechanical small bowel obstructions, its significance raises tremendously in the elderly population where it accounts of up to a quarter of all small bowel obstructions[32]. First described by Bartholin in 1654[34,37], this syndrome gained attention with Dr. Rigler's classic paper in JAMA in 1941[69]. Typical pathogenesis of gallstone ileus begins with acute or chronic cholecystitis, often in the setting of cholelithiasis, which spreads inflammation and adhesion to the alimentary tract, leading to development of a biliary-enteric fistula. Given the anatomic proximity of the duodenal wall, cholecystoduodenal fistula accounts for overwhelming majority (86%-96%) of the tracts, followed by cholecysto-colonic, cholecysto-gastric,

and choledocho-duodenal fistulas[33,34,57,69-74]. Gallstones eventually enter the intestinal tract and may result in clinical obstruction with classic predilection to known areas of stasis in the physiologically narrower distal ileum and ileocecal valve (in 60% to 85% of the cases), proximal small intestine, stomach, and finally colon[34,37,38]. Chemical analysis of the composition of enterolith reveals it gallstone origin; however, secondary depositions may occur in the long standing occult disease and vary by location. Size of the gallstones typically varies between 2.5 to 4.5 cm, but may achieve significantly large dimensions of 10 cm in some patients[34,69]. Reactive substances in the bile juice found in the gallstones may react with intestinal epithelial cells, potentially leading to significant mucosal injuries.

Finally, bowel obstruction secondary to migrated renal stone is extremely rare, but has been previously described in patients with significant reno-enteral adhesions and fistulization[40,41].

CLINICAL PRESENTATION

Clinical presentation of enterolithiasis varies according to the etiology, age, location, chemical composition, finally, dimensions of the stone. Primary enterolithiasis should be suspected in a younger patient with underlying inflammatory bowel disease (industrialized countries) or tuberculosis (third world countries) or an older patient with intestinal surgery or small bowel diverticular disease who presents with abdominal pains, distention, nausea, and vomiting of occasionally sudden but often fluctuating subacute nature which occurs as a result of the enterolith tumbling through the bowel lumen[11,28,43]. Fevers or chills may be present and physical examination may be remarkable for attenuated bowel sounds, tympany, and abdominal tenderness. Laboratory analysis may reveal leukocytosis and anemia, both from the underlying disease and enterolith related pressure on the intestinal mucosa. Elevated C-reactive protein and erythrocyte sedimentation rate may be present. Rarely a patient may present with a bowel perforation.

Patients with gallstone ileus behave similarly. Abdominal pain is seen in over 90% of the patients with gallstone ileus, followed by vomiting in 60%-95%, abdominal distention in 54%-84%, and constipation in 54.5%, fever in 41%, jaundice in 7% of the cases[33,34]. Female and geriatric patient predilection may be an early clue and history of gallbladder disease may be present in 27%-50% of the patients. Pre-existing comorbidities including cardiovascular, respiratory, and renal diseases are common, and concomitant malignancy may be present[34,39].

MIMICKERS OF ENTEROLITHIASIS

Non-specificity of the symptoms of enterolithiasis and its rarity may lead to delay in effective diagnosis and management. In absence of clinical suspicion, the differential diagnosis is typically wide and may include common causes of bowel obstruction (hernia, adhesions, inflammatory/infectious conditions, tumors, and intussusception), diverticulitis, appendicitis, duodenitis, peritonitis, pancreatitis, and peptic ulcer disease. Intra-luminal swallowed foreign bodies and extra-luminal calcified pathology including phleboliths, ureteral stones, and lymphadenopathy may erroneously lead to misdiagnosis of enterolithiasis. Biliary and renal calculi, mesenteric teratoma, fat necrosis, calcified fibroids, fecaliths, calcified epiploic appendages, and omental calcifications may confound the clinical diagnosis[4,15,20,43]. Finally, clinically insignificant incidentally noted enteroliths may shift attention from primary non-enterolith related pathology of the patient that may be responsible for a current clinical presentation.

CLINICAL DIAGNOSIS

Detailed history and physical examination are necessary in evaluation of a patient with suspected enterolithiasis. Correct diagnosis is established in appropriate clinical setting after excluding other common pathologic processes. A history of sudden or recurrent abdominal pain, associated with vomiting in a patient who is in a population at risk for enterolithiasis should raise suspicion of a possibility of enteral concretions. Symptom review may help identify patients with acute or indolent disease. Historically, diagnosis of enterolithiasis was made at the time of laparotomy or autopsy. Development of radiological field has tremendously improved early diagnosis and treatment of this disorder. Presence of single or multiple enteroliths on imaging is helpful in establishing correct diagnosis. Mobile nature of the stones and their anatomic location may lead to differentiating between various underlying pathologies, including intestinal strictures/stenosis in infectious or inflammatory bowel disease, post-surgical complications, or Meckel's diverticular disease. Traditionally, plain abdominal roentgenograms are the first step in identifying enteroliths and can detect stones in up to a third of the cases[8]. The visibility of the stone depends on the calcium content, with enteroliths containing a higher proportion calcium salts being more radiopaque and forming in the relatively more alkaline environment of the distal ileum. Choleic acid enteroliths are more radiolucent and form in the more acidic environment of the proximal small bowel[15,43,45]. Clinician's awareness of anticipated chemical composition of the enterolith in a particular bowel segment, will increase the yield of radiologic detection and potentiate proper diagnosis. Important radiographic features of enteroliths include dense rim with pale core in oval, round, or rectangular shadows, "coin-end-on" appearance, and apparent mobility on serial examinations in relation to each other and to a fixed anatomical pathology[75]. Computed tomography (CT) scan with oral contrast may provide two or three dimensional orientation and increase the yield of detection of radiolucent stones. CT scan may also help in identifying the number of enteroliths, their exact location, and narrow the focus on the culprit

stone. Dedicated radiologic imaging may assist in establishing underlying pathology of the intestinal tract that leads to a stone formation or is responsible for stone trapping and clinical obstruction. Diagnosis of small intestinal Crohn's disease, diverticulosis, tumor, anastomotic stricture, fistulizing disease, regional enteritis, altered anatomy and blind loops may provide additional clues in patient care. Finally, particular attention should be directed to the gallbladder and biliary system to rule out gallstone ileus.

Imaging of patients with Meckel's enterolithiasis may suggest dilated small bowel with air fluid levels in setting of an obstruction in 40% of the cases on plain abdominal radiographs, single or multiple opaque stones in 88% of the patients. Location of the stones may vary, with nearly 60% present in the right lower quadrant. Majority of the stones have peripheral calcifications with radiolucent centers (89%) and range from 1 to 5 cm in diameter[29]. Yield of enterolith and Meckel's diverticulum detection increases with contrast studies, with both CT enterography and small bowel series/enteroclysis complementing plain film radiography and providing additional advantage in identifying underlying pathology. Nuclear studies aimed at detecting ectopic gastric tissue in the Meckel's diverticulum may be helpful but given the high incidence of symptomatic presentation of such diverticula in childhood that is followed by surgical resection, may not always be diagnostic in older patients who have intestinal tissue lining of the diverticulum. It may, however, provide additional information in select rare cases of radiolucent stones that may be formed within acidic environment of gastric mucosal Meckel's and are not visible on plain roentgen films or a CT scan. Extrusion of the enterolith into the small bowel from the diverticulum may lead to the enteric occlusion and alternatively, distention of the Meckel's diverticulum from the large enterolith may cause Mirizzi-type impression on the adjacent gut, both resulting in signs of small bowel obstruction on CT scan[76].

Gallstone ileus represents a better known category of enterolithiasis. Clinical diagnosis should be suspected in a patient who presents with vague complains of intermittent abdominal distention, pains, nausea and vomiting due to "tumbling phenomena" of the stone passage though the intestinal tract[38]. Additional findings may include weight loss, dehydration, and loss of appetite[77]. Duration of symptoms may vary from days to weeks and the diagnosis is typically delayed for several days. Correct preoperative diagnosis is achieved in only half of the cases[78]. A quarter of cases will have an antecedent gallstone disease[34]. Classic description by Leo Rigler in 1941 of pneumobilia or contrast medium in the biliary tract, partial or complete small bowel obstruction, and a visualized ectopic gallstone that may change position within the bowel (Rigler's triad)[69] is seen in less than 50% of patients with gallstone ileus[31,34,36,38,57,69,73,79,80]. Combined radiologic imaging (X-rays, ultrasound, CT scan) result in increased yield of diagnosis, with positive findings in nearly 80% of the patients[34].

Importantly, colloquial information obtained from radiologic diagnosis of enterolithiasis may provide an important information on the location of the underlying intestinal pathology, including stenotic segments of Crohn's disease, areas of enteric tumors, significant adhesions, incidental Meckel's diverticulum, and hernias.

Notably, with the advent of radiologic procedures and rapid growth of the medical field, there exists a wider gap in the studies addressing potential increase in prevalence of enteroliths today compared to the mid-late 20th century. Certainly, higher detection rate may aid in differential diagnosis of a number of intra-abdominal pathologies, including establishing early clue of enterolithiasis. However, the definitive diagnosis of an intestinal stone is made through removal of the endolith and its subsequent pathology.

COMPLICATIONS AND PROGNOSIS

Complications related to enterolithiasis should be sought for and recognized early. *De novo* formation and subsequent transit of an enterolith through the gastrointestinal tract may result in acute, subacute, or chronic, intermittent, partial or complete intestinal obstruction[76]. Important risk factors include intraluminal stricturing or stenosis seen in inflammatory bowel disease, tuberculous and radiation enteritis; surgical anastomoses; intestinal malignancy; extraluminal kinking or angulation found in the setting of intra-abdominal adhesions, external compressions, or incarcerated hernias; and finally abnormally narrowed intraluminal diameter in otherwise unremarkable terminal ileum and highly patent ileocecal valve. It is generally accepted that, in the absence of mechanical or structural luminal compromise, stones larger than 2.5 cm in diameter may cause an intestinal obstruction[33]. Single enteroliths smaller than 2 cm in size would typically pass unnoticed through normal small intestine and into the colon; but if retained, may become a nidus for additional calcification and growth and may result in pathogenic obstruction in the future. Impacted enterolith may incite direct pressure injury to the intestinal mucosa, potentially worsened by chemical damage from the reactive substances found on its shell[33]. Intestinal gangrene in association with enterolith has been previously reported[7]. Additional rare complications of enterolithiasis include intussusception of small bowel[81], acute obstructive ascending cholangitis due to periampullary duodenal stone[82], afferent loop syndrome[23,83], diverticulitis[63,84], iron deficiency anemia[85], gastrointestinal hemorrhage[45], and perforation[7,16,19,86,87]. Mortality of uncomplicated primary enterolithiasis is very low, but may rise to 3% in the poorly conditioned patients with significant obstruction and delay in diagnosis[42]. Morbidity from the gallstone ileus in the second half of the twentieth century was reported by Reisner and Cohen[38] to include wound infection in 32% of cases, biliary symptoms in 15%, and recurrence in 5% - all

likely to continue to improve with advances in today's medical care, whereas mortality remained high at 18%. Review of the more recent Japanese literature projects a decrease in mortality to 8%[33]. Continued improvement in medical diagnosis and treatment of enterolithiasis will effectively allow for a steady decrease in its associated complications and overall mortality.

TREATMENT

Optimal treatment of enterolithiasis should focus on enterolith removal and correction of underlying pathology to prevent future formation of additional enteroliths. In cases of acute intestinal obstruction, expectant management with serial abdominal examinations, electrolyte correction, appropriate hydration, and nasogastric tube suctioning may be selectively considered for stones less than 2 cm in size in absence of underlying luminal compromise[88]. Spontaneous passage of a larger stone is unlikely and a thorough search for an underlying pathology should be performed. In cases of intestinal stricturing, stenosis, or an anastomotic defect, an attempt at endoscopic segment dilatation and stone retrieval may be considered first[12,89]. Endoscopic electrohydraulic lithotripsy and mechanical lithotripsy have been previously described[84,90]. Surgical management remains the mainstay of therapy in the majority of the cases, with an attempt at digital fragmentation of the stone followed by manual "milking" of the smaller parts into the large intestine being successful in nearly 50% of the cases[9,42]. Alternatively, proximal enterotomy of the non-edematous segment with manual enterolith removal may be performed. Preoperative percutaneous decompression of the afferent limb using ultrasound guidance may be indicated in Billroth II patients[23]. Segmental small bowel resection with intended primary anastomosis should be attempted in the setting Meckel's diverticulum, long complicated strictures, diverticulitis, significant inflammation, intestinal necrosis, perforation, and enteral duplication[20,75]. Most cases described in the literature have been open procedures, although Jones *et al*[76] and Shah *et al*[91] report two cases in which resection was successfully performed laparascopically. This approach decreases the detection rate of additional enteroliths that may need to be sought for and eliminated by milking of the proximal bowel to decrease recurrence of obstruction. Three cases have been reported where surgical removal of Crohn's disease associated enteroliths revealed adenocarcinoma of the bowel[43,45,55], therefore raising awareness for intraoperative evaluation for small bowel tumors.

The procedure of choice in gallstone ileus is still a matter of controversy with possible approached including enterolithotomy alone, in conjunction with simultaneous cholecystectomy and fistula closure, or a two-stage procedure. Higher morbidity and mortality seen in the longer one-stage procedure compared to enterolithotomy alone has led to the latter being the preferred approach in the emergency setting in many centers[34]. However, persistence of biliary-enteric fistula may lead to recurrent gallstone ileus (5%) or cholangitis (11%)[34,57]. Therefore, surgical options should be individualized and if general medical condition of a patient permits, one-step enterolithotomy with cholecystectomy and fistula closure may be considered.

Importantly, enterolith formation may be the first clue to the existence of a compromised intestinal anatomy and every effort should be made to decrease future stone formation by recognizing and treating underlying medical conditions. Medical, endoscopic, or surgical correction of inflammatory, infectious, or structural pathology may provide chronic symptom relief and benefit the long term outcome in many of the cases.

With rapid advances in medical and surgical technology and procedural skills, additional studies are needed to assess the success rate of new approaches to the removal of enteroliths in the twenty first century. Single- and double-balloon enteroscopy with carbon dioxide insufflation may provide additional benefit to the selected patients that were previously managed surgically. This tactic may potentially result in the future shift from surgical into the endoscopically feasible realm, thus decreasing morbidity and mortality associated with surgical intervention and improving patient's outcome.

Finally, "silent" enterolithiasis may occur at increasing rates in the era of radiologic advances. A single or clustered stones may be incidentally visualized in the areas of known diverticulosis including Meckel's diverticulum or strictures/stenosis. Alternatively, finding of an enterolith may an early clue to underlying pathology and further clinical evaluation may be warranted. Incidental enteroliths may potentially lead to complications and will therefore require periodic re-assessment. Endoscopic or surgical retrieval may be considered in select cases.

CONCLUSION

Enterolithiasis remains an important clinical condition with raising incidence and prevalence. Alterations in bowel anatomy and microenvironment play a significant role in pathogenesis of this disease and provide an important clue in its etiologic recognition, chemical classification, and clinical presentation. Distinction between primary and secondary enterolithiasis and identification of underlying enteropathy is crucial in establishing disease process. Clinical diagnosis relies on detailed history and physical examination complemented by radiologic imaging modalities. Mimickers of enterolithiasis should be diligently excluded. Treatment should be aimed at endoscopic or surgical enterolith removal and correction of the underlying intra- and extra-intestinal pathology to prevent additional stone formation. Rapid advances in medical field will continue to lead to improved diagnosis and help expand therapeutic options for the affected patients.

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Dendritic cells in hepatitis C virus infection: Key players in the *IFNL3*-genotype response

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Abstract

Recently, single nucleotide polymorphisms, in the vicinity of the interferon lambda 3 (*IFNL3*) gene have been identified as the strongest predictor of spontaneous and treatment induced clearance of hepatitis C virus (HCV) infection. Since then, increasing evidence has implicated the innate immune response in mediating the *IFNL3* genotype effect. Dendritic cells (DCs) are key to the host immune response in HCV infection and their vital role in the *IFNL3* genotype effect is emerging. Reports have identified subclasses of DCs, particularly myeloid DC2s and potentially plasmacytoid DCs as the major producers of IFNL3 in the setting of HCV infection. Given the complexities of dendritic cell biology and the conflicting current available data, this review aims to summarize what is currently known regarding the role of dendritic cells in HCV infection and to place it into context of what is know about lambda interferons and dendritic cells in general.

Key words: Hepatitis C virus; Interferon lambda 3; Dendritic cells; Plasmacytoid dendritic cells; Myeloid dendritic cells; Innate immunity

Core tip: Increasing evidence implicates the innate immune response in mediating the interferon lambda 3 (*IFNL3*) genotype effect in hepatitis C virus (HCV) infection. Dendritic cells (DCs) are essential players in the host immune response to HCV infection, especially with respect to the *IFNL3* genotype effect. Subsets of DCs, myeloid DC2s and potentially plasmacytoid DCs, appear to particularly important in orchestrating the *IFNL3* genotype effect.

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INTRODUCTION

An estimated 3% of the world's population is infected with hepatitis C virus (HCV)[1]. With low spontaneous clearance rates, 80% of individuals go on to develop chronic infection, which is associated with long term complications including cirrhosis, hepatocellular carcinoma and death from chronic liver failure[2]. Recently, single nucleotide polymorphisms (SNPs) in the region of the interferon lambda 3 (*IFNL3*; formerly known as *IL28B*) gene were identified to strongly predict spontaneous and treatment-induced clearance of HCV of

HCV infection[3-7]. *IFNL3* encodes IFNL3 a member of the type Ⅲ interferon (IFN) family and thus belongs to the group of innate immune cytokines. Dendritic cells (DC) are recognized as the major producers of IFNs and central players in the host immune response against HCV[8]. In this review we explore the role of DC in chronic hepatitis C (CHC) in the context of IFNL3 and its polymorphism.

IFNL3 POLYMORPHISMS IN HCV INFECTION

The poor treatment response rates, high economic burden and significant adverse effects associated with traditional antiviral therapy for CHC consisting of pegylated IFN-alpha and ribavirin (Peg-IFNα/RBV) motivated research into host genetic factors associated with successful HCV clearance. In 2009, four landmark genome wide association studies (GWAS) independently described SNPs in the vicinity of *IFNL3* that were dramatically predictive of response to Peg-IFNα/RBV therapy in patients with genotype 1 HCV[3-5,9]. The favourable variants of the two most widely studied SNPs, *rs12979860* and *rs8099917*, are the strongest pre-treatment predictors of SVR in genotype 1 HCV infection, but clearly also affect treatment response to Peg-IFNα/RBV in HCV genotype 2 and 3 infections[10]. Subsequently, this genetic variation has also been associated with spontaneous clearance of HCV[6,7] and liver inflammation in chronic HCV infection[11-13], strongly implicating the innate immune response in the *IFNL3* genetic response.

LAMBDA INTERFERONS

Three classes of IFNs are now recognized (type Ⅰ, Ⅱ and Ⅲ) and these cytokines are crucial to the establishment of an antiviral immune response. They are classified based on differences in structure, receptor and biological function: Type I IFNs include IFNA and IFN-beta (IFN-β), whereas the only type Ⅱ IFN is IFN-gamma (IFN-γ)[14]. The type Ⅲ or lambda IFNs were more recently identified in 2003 by two independent research groups[15,16]. Initially three members in this family were described: IFNL1 or IL29, IFNL2 or IL28A and IFNL3 or IL28B. Interestingly, lambda IFNL share similarities with both the IL10 family of cytokines and type I IFNs[17]. They signal through the same janus tyrosine kinase (JAK)/signal transducers and activators of transcription (STAT) pathway leading to induction of interferon-stimulated genes (ISGs) and antiviral activity[15,18]. Signaling through common pathways facilitates type I and type Ⅲ IFNs to induce similar biological activities, mediated by the induction of nearly identical sets of more than 300 ISGs[19]. Lambda IFN induced antiviral activity has been reported against many different viruses including inhibition of HCV replication *in vitro*[19,20]; IFNL3 has been shown to inhibit HCV replication in three independent HCV models by the JAK/STAT pathway[21]. There is also *in vitro* evidence that IFNA induces expression of IFNL genes and that both cytokines appear to enhance the activity of the other[22].

In addition to their antiviral properties, lambda IFNLs exert complex and varied effects on immune cell function that are likely context dependent: Briefly, lambda IFNs have been shown to reduce the production of T helper 2 (Th2) cytokines (IL-4, IL-13, IL-14 and IL-15) thus potentially favouring Th1 driven immune response[23,24]. Further, enhanced adaptive immunity has been suggested by IFNL3 induced reduction in regulatory T cells, increased $CD8^+$ T cell numbers[25] and augmentation of $CD8^+$ T cell cytotoxicity[26].

In 2013, a new polymorphism (*rs368234815*) located between the genes for *IFNL2* and *IFNL3* (Figure 1) was identified and found to induce a frame shift mutation resulting in transient expression of an IFN analogue, IFN-lamda 4 (*IFNL4*), in stimulated human hepatocytes[27]. The authors postulated that the genotype dependent production of the protein IFNL4 resulted in altered ISG expression and thus may explain the effects on viral clearance. *rs368234815* is in high linkage disequilibrium with *rs12979860*, but more strongly associated with spontaneous and treatment induced HCV clearance, especially in individuals of African ancestry. The exact mechanisms molecular functions of IFNL4 remain to be clarified[28,29].

INTERFERON LAMBDA RECEPTOR AND SIGNALING

All IFNLs signal through the same heterodimeric receptor complex. This is composed of a unique interferon lambda receptor 1 (IFNLR1) [also known as IL28R alpha or cytokine receptor family 2 member 12 (CRF2-12)] component and IL10R2[15,16]. Both subunits are required for signalling. IL10R2 is ubiquitously expressed while IFNLR1 displays restricted tissue expression, predominately on cells of epithelial origin (including: keratinocytes and cells of kidney, lung and gastrointestinal tract origin) as well as specific subsets of immune cells[30-32]. The *IFNLR1* gene generates several splice variants including: a full length, membrane bound IFNLR1 and secreted, soluble IFNLR1 protein[33].

Expression of *IFNLR1* mRNA in human immune cells, especially B, T and NK-cells, has been previously demonstrated[30]. However, these immune cells were shown to express relatively more soluble receptor, which is postulated to act as an inhibitor to IFNL activity[30]. In contrast to these reports high levels of IFNLR1 have been detected on plasmacytoid dendritic cells (pDCs) relative to other cell populations in peripheral blood mononuclear cells (PBMCs) by us and others[30,34,35]. Furthermore, we have demonstrated significant up-regulation of *IFNLR1* expression after IFNA stimulation in pDCs suggesting that IFNA may enhance IFNL receptor expression and sensitivity to IFNL. Analysis of the ratio of membrane-bound receptor (*IFNLR1*-mem) to soluble isoforms (*IFNLR1*-sol) for pDCs, demonstrated that the majority

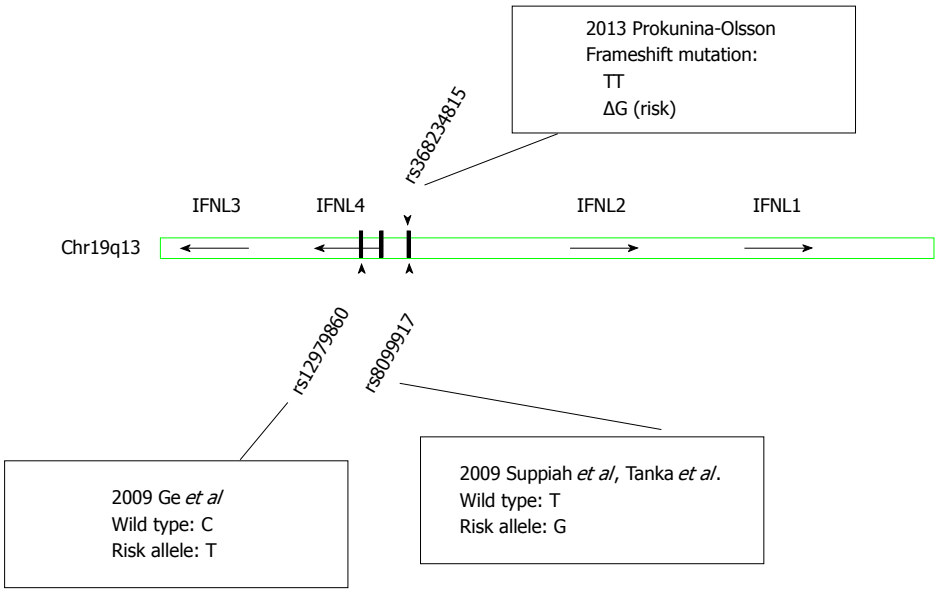

Figure 1 Schematic representation of single nucleotide polymorphisms idenditied in the interferon lambda gene locus. IFNL: Interferon lambda.

was the isoform encoding the membrane-associated or functional form of IFNLR1[36]. We have also demonstrated that *IFNLR1* expression was not significantly higher in HCV-infected liver biopsies compared with unstimulated pDCs[36].

Previous work has produced conflicting evidence on whether or not immune cells are a target for Type Ⅲ IFNs. Several studies have failed to show a response to IFN-lambdas (IFNL1 and/or IFNL2) by a variety of immune cells including B, T and natural killer cells (NK cells) as well as monocytes[30,37]. In contrast, several other human studies have revealed a direct effect of IFNLs on monocytes[38,39], dendritic cells[37] and T cells[23,38,40]. Work in pDCs has shown that IFNL1 results in altered expression of costimulatory molecules such as CD80[41]. We and others have demonstrated that pDCs are responsive to IFNL3 as detected by up-regulation of the ISG *MxA*[36] and increased production of IFNα[42]. IFNL may indeed act as an autocrine signal for pDCs with the ability to improve survival and enhance antiviral response[35]. This suggests a positive feedback loop for the production of IFNL3, particularly by DCs within HCV infected livers and the potential for an augmented response with IFNA therapy.

DENDRITIC CELLS

DCs are professional antigen presenting cells and play a major role in orchestrating the innate immune response against hepatitis C virus[43]. They are a rare cell population representing 0.3%-0.5% of normal human peripheral blood mononuclear cells[44,45]. They can be broadly categorized into two major subsets: pDCs and conventional myeloid DCs (mDCs). pDC and mDc differ significantly in terms of their morphology, phenotype and function; their individual features are summarized in Table 1.

mDCs originate from myeloid precursors in the bone marrow and display classic DC morphology with branched protrusions or dendrites[46]. mDCs are classical antigen presenting cells and have the ability to activate naive and effector T cells[43]. mDC can be further subdivided into mDC1 CD1c$^+$ (blood antigen 1$^+$; BDCA1$^+$) or mDC2 CD141$^+$ (blood antigen 3$^+$; BDCA3$^+$)[47]. Human mDC2s are reported to be a counterpart of murine CD8a$^+$ DC[48]. mDCs express a variety of toll like receptors (TLRs) such as TLR2 recognizing viral ligands (including HCV core and NS3) and TLR3 recognizing double stranded RNA viruses[49]. mDC2s express higher levels of TLR3 than mDC1s and lack TLR4 expression. mDC2s are the rarest DC population in bone marrow and peripheral blood[50]. mDC1s are the most potent producers of IL-12 thus rendering them more efficient than mDC2s at promoting cytotoxic CD8$^+$ T-cell responses[51].

In contrast, pDC display a plasma cell morphology and under steady state conditions express lower levels of MHC class I, MHC class Ⅱ and co-stimulatory molecules such as CD86[8]. pDCs strongly express the pattern recognition receptors, TLR7 and TLR9, but not TLR3 and are thus capable of recognizing single stranded RNA and unmethlyated CpG-containing DNA ligands respectively[52]. Upon exposure to viral stimuli they are well recognized to produce massive amounts of type I interferons and acquire the capacity to present antigen[53]. In addition, they provide help to natural killer cells[54], regulate cell trafficking through the production of chemokines[55] and alter Th1/Th2 responses[56].

In CHC numbers of circulating pDCs and mDCs are reduced in peripheral blood compared with healthy controls[57-61] but both populations of DCs are significantly increased in the livers of CHC patients[61,62]. Furthermore in CHC, circulating numbers of DCs are inversely correlated with the serum alanine aminotransferase levels and severity of liver disease[63]. This suggests that immune cell trafficking to the liver may be the reason for reduced pe-

Table 1 Subsets of human dendritic cells

	Plasmacytoid dendritic cells	Myeloid dendritic cells	
Morphology	Round, resemble plasma cells	Prominent cytoplasmic protrusions	
Phenotype	$CD11C^-$ $CD1a^+$ $CD123^{high}$	$CD11c^+$ $CD1a^+$ $CD123^{low}$	
		mDC1	mDC2
	$BDCA\text{-}4^+$ ($CD304^+$)		
	$BDCA\text{-}2^+$ (CLEC4C)	$BDCA\text{-}1^+$ ($CD1c^+$)	$BDCA\text{-}3^+$ ($CD141^+$) CLEC9A
TLR receptor expression	TLR1, TLR6, TLR7, TLR9, TLR10	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8,TLR10	TLR1, TLR2, TLR3,TLR6, TLR8, TLR10
MHC Ⅰ	+	++	++
MHC Ⅱ	+	+++	++
CD86	+	+++	++
CD40	+	++	+++
CXCR3	+++	+	++
ICOS L	++	+	+++

mDC: Myeloid dendritic cell; BDCA: Blood dendritic cell antigen; TLR: Toll like receptor; MHC: Major histocompatibility complex; CXCR3: Chemokine receptor 3; ICOSL: Inducible costimulator ligand.

ripheral DCs numbers. Enriched mDC2 numbers in CHC infected livers have also recently been demonstrated[42,64]. Apart from enrichment in the liver, hepatic mDC2s display higher expression of CD40, CD80, CD83 and CD86 than those seen in the circulating peripheral blood compartment suggesting a more mature phenotype[65].

DCS AND HOST IMMUNE RESPONSE TO HEPATITIS C VIRUS

Both the innate and adaptive arms of the immune system contribute to the host's ability to resolve HCV infection. The first line of defense against viral infections is the innate immune response with the IFNs playing a key role in induction of the antiviral state and control of HCV replication[66]. Specific viral motifs known as pathogen-associated molecular patterns are recognized by pattern recognition receptors (PRRs). Two groups of PRRs sense viral infection, RIG-I like-receptors and TLRs (TLR3, 7, 8 or 9)[67]. Downstream signalling leads to translocation of IFN regulatory factor 3 and synthesis of IFNs and pro-inflammatory cytokines[68].

Human pDCs recognize HCV predominantly through a TLR7 medicated pathway[59]. mDCs recognize HCV infection and mediate IFNL induction by the dsRNA sensing, TLR3-mediatd pathway[42]. Subsequently secreted IFNs bind to the IFN receptors and activate the JAK/STAT pathway leading to the induction of ISGs[69]. The expression of ISGs establishes an antiviral state including in neighboring uninfected hepatocytes. However, the induction of the endogenous IFN system in the liver has limited antiviral efficiency with, persistence of HCV observed for decades despite the expression of hundreds of ISGs[70,71] In fact, it is now well established that patients with an activated endogenous IFN system are poor responders to IFNA based therapies[70-72]. Interestingly, there is evidence that hepatic IFNL rather than type I IFN induction is more closely correlated with the strength of the ISG response[73].

HCV has the ability to impede the IFN response at several levels including: NS3/4A protein cleaving adapter molecules and blocking PRR signalling; HCV core protein interfering with JAK/STAT signalling and ISG expression; NS5A inhibiting the function of several ISGs and HCV may interact directly with pDC to impair IFN production and promote apoptosis[74]. In this context, HCV has the ability to evade the host antiviral response in hepatocytes through cleavage of key molecules involved in RIG-I and TLR3 signalling hence, interfering with the induction of endogenous IFNs and ISGs. pDC by contrast have the ability to overcome this evasion through direct cell contact and transfer of viral RNA from hepatocytes recognized by TLR7. This leads to the synthesis of interferon stimulated genes and secretion of IFNs[59]. There is some evidence that in CHC infection DC function is impaired as a result of reduced antigen presentation to $CD4^+$ T cells mediated through interference by HCV proteins[43].

DENDRITIC CELLS ARE THE MAJOR PRODUCERS OF INTERFERON LAMBDA

There is evidence that human hepatocytes, DCs and macrophages all produce IFNL in response to HCV infection[41,75-79]. Importantly, mDC2 peripheral blood DCs have been identified by several groups as major producers of IFNL in HCV infection[42,65]. Data suggests that IFNL induction is dependent on direct cell to cell interaction with HCV infected hepatoma cells mediated through TLR3 signalling[42]. In comparison to other DC subsets mDC2s produced large amounts of IFNL when stimulated with cell-cultured HCV and HCV-transfected Huh7.5.1 cells[65]. This is further supported by evidence that the mouse homologue for human mDC2, $CD8\ alpha^+$ cells, are potent producers of IFNL2 and IFNL3 following TLR3 activation[80].

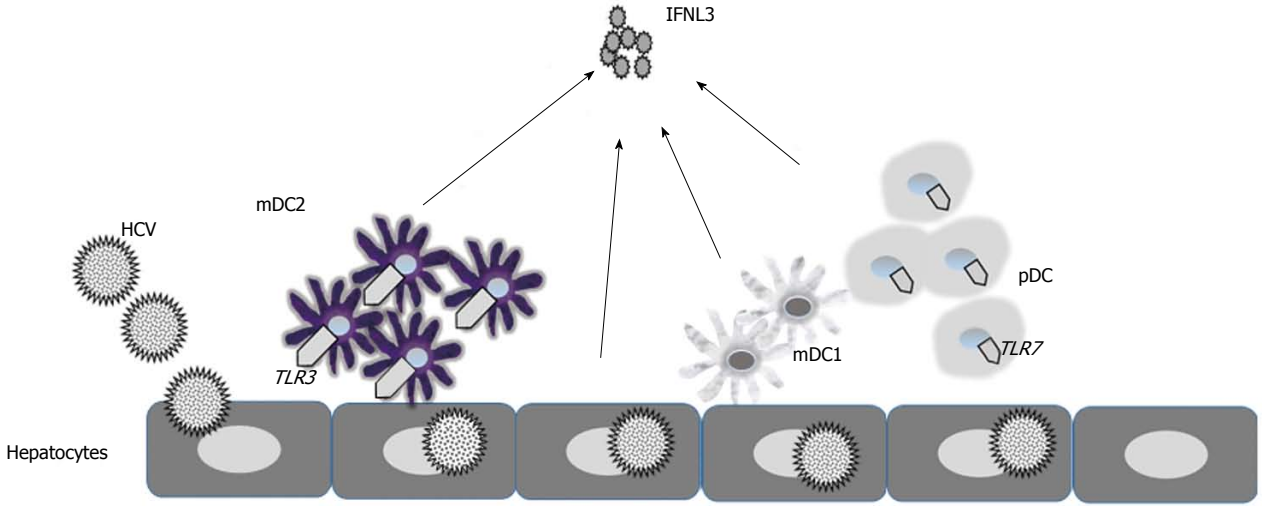

Figure 2 Schematic representation of interferon lambda 3 production in hepatitis C virus infection. Dendritic cell populations are enhanced in hepatitis C virus (HCV) infected livers. In response to HCV infection hepatocytes and dendritics produce interferon lambda (IFNL). However, recent reports suggest myeloid dendritic cell 2 (mDC2) are the major producers of IFNL3. IFNL3 production is mediated *via* toll like receptor (TLR)3 in mDC2 and TLR7 in plasmacytoid dendritic cell (pDC).

In contrast to these studies Murata *et al*[81] found that mDC2s stimulated by TLR3 agonists and pDCs stimulated by TLR7 agonists both produce large amounts of IFNL3. Importantly, detectable levels of IFNL3 were only demonstrated by TLR7 agonists and not TLR3 agonists in PBMCs. There was evidence of a more robust production of *IFNL3* mRNA in PBMC of patients with hepatitis C with the favorable *IFNL3* genotype (*rs8099917* TT) after stimulation with TLR7 agonists[81]. Importantly, this study detected that induction of IFNL3 protein was strongly correlated with Peg-IFNα/RBV treatment response in CHC[81] and that this measurement was a more accurate predictor of treatment response (95.7%) than *IFNL3* genotyping (65.2%)[81] (Figure 2).

To date, the literature has been conflicting as to whether *IFNL3* genotype alters *IFNL3* expression. Early work suggested higher expression in whole blood in the responder genotype[4,5]. However, similar studies in PBMCs did not confirm this association[3,42]. In several independent reports examining liver biopsies from subjects with CHC, no association between *IFNL3* expression and *IFNL3* genotype was noted[82-84]. Yoshio *et al*[65] identified greater IFNL3 production by mDC2s of patients with *IFNL3* responder genotypes *in vitro* with HCV co-culture but not TLR3 agonists. Other reports have found no *IFNL3* genotype association with IFNL production in mDC2s[42] or pDCs[36]. Thus, it is possible that IFNL3 production is temporally regulated, cell type and context dependent and that genotype differences may only be observed in particular cell populations at crucial stages of infection. Further complicating DC analysis is evidence that peripheral blood DC differ from tissue resident DC[85,86].

CONCLUSION

Since the identification *IFNL3* polymorphisms as important predictors of HCV clearance in 2009, signficant progress has been made relating to the underlying mechanisms: Particularly, dendritic cell subsets such as mDC2s and potentially pDCs seem to be important to IFNL3 phenomena. Given their pivotal roles in innate and adaptive immunity, genetically determined, differential regulation of IFNL3 expression is thus likely to control disease outcomes. It has remained difficult, however, to pinpoint exactly, how the polymorphism translates into different regulation. Further research is required to clarify the genetic association with clinical outcomes and how IFNL3 medicated DC responses change the ultimate outcome of HCV infection.

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ORIGINAL ARTICLE

Elevated free cholesterol in a p62 overexpression model of non-alcoholic steatohepatitis

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Abstract

AIM: To characterize how insulin-like growth factor 2 (*IGF2*) mRNA binding protein p62/IMP2-2 promotes steatohepatitis in the absence of dietary cholesterol.

METHODS: Non-alcoholic steatohepatitis (NASH) was induced in wild-type mice and in mice overexpressing p62 specifically in the liver by feeding the mice a methionine and choline deficient (MCD) diet for either two or four weeks. As a control, animals were fed a methionine and choline supplemented diet. Serum triglycerides, cholesterol, glucose, aspartate aminotransferase and alanine transaminase were determined by standard analytical techniques. Hepatic gene expression was determined by real-time reverse transcription-polymerase chain reaction. Generation of reactive oxygen species in liver tissue was quantified as thiobarbituric acid reactive substances using a photometric assay and malondialdehyde as a standard. Tissue fatty acid profiles and cholesterol levels were analyzed by gas chromatography-mass spectrometry after hydrolysis. Hepatocellular iron accumulation was determined by Prussian blue staining in paraffin-embedded formalin-fixed tissue. Filipin staining on frozen liver tissue was used to quantify hepatic free cholesterol levels. Additionally, nuclear localization of the nuclear factor kappa B (NF-κB) subunit p65 was examined in frozen tissues.

RESULTS: Liver-specific overexpression of the insulin-like growth factor 2 mRNA binding protein 2-2 (IGF2BP2-2/IMP2-2/p62) induces steatosis with regular chow and amplifies NASH-induced fibrosis in the MCD mouse model. Activation of NF-κB and expression of NF-κB target genes suggested an increased inflammatory response in *p62* transgenic animals. Analysis of hepatic lipid composition revealed an elevation of monounsaturated fatty acids as well as increased hepatic cholesterol. Moreover, serum cholesterol was significantly elevated in *p62* transgenic mice. Dietary cholesterol represents a critical factor for the development of NASH from hepatic steatosis. Filipin staining revealed increased free cholesterol in *p62* transgenic livers, which were not diet-derived. The mRNA levels of the rate-limiting enzyme for cholesterol synthesis 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase or HMGCR) were not significantly upregulated, potentially due to increased cholesterol biosynthesis *via* elevated sterol regulatory element binding transcription factor 2 (*SREBF2*) gene expression and increased iron

deposition in transgenic animals.

CONCLUSION: This study provides evidence that p62/IGF2BP2-2 drives the progression of NASH through elevation of hepatic iron deposition and increased production of hepatic free cholesterol.

Key words: Insulin-like growth factor 2 mRNA binding protein 2-2; Methionine/choline deficient; Non-alcoholic fatty liver disease; Filipin; Iron

Core tip: Dietary cholesterol represents a critical factor for the development of non-alcoholic steatohepatitis (NASH) from steatosis. Liver-specific overexpression of the insulin-like growth factor 2 mRNA binding protein p62/IMP2-2/IGF2BP2-2 induces steatosis and amplifies NASH-induced fibrosis. Here, we show that p62 elevates monounsaturated fatty acids and hepatic cholesterol in the absence of exogenous cholesterol. Filipin staining demonstrates increased free cholesterol in *p62* transgenic livers. Srebf2-induced cholesterol biosynthesis in transgenics is most likely due to pronounced hepatic iron accumulation, which is also associated with lipid peroxidation in transgenic livers. In summary, p62/IGF2BP2-2 drives the progression of NASH by increasing hepatic free cholesterol.

Simon Y, Kessler SM, Gemperlein K, Bohle RM, Müller R, Haybaeck J, Kiemer AK. Elevated free cholesterol in a p62 overexpression model of non-alcoholic steatohepatitis. *World J Gastroenterol* 2014; 20(47): 17839-17850 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17839.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17839

INTRODUCTION

In industrialized countries, non-alcoholic fatty liver disease (NAFLD) represents the most frequent hepatic manifestation of chronic liver diseases, whereby hepatic steatosis is the first hit sensitizing the liver to a second hit that ultimately leads to hepatocyte injury, inflammation, and subsequent fibrotic changes[1]. Prolonged NAFLD can lead to hepatocellular carcinoma (HCC)[1], which is the sixth most common malignancy worldwide and the second leading cause of cancer-related deaths[2]. However, the pathophysiological mechanisms leading to the progression from NAFLD to end stage liver disease are still poorly understood.

The composition of fatty acids in the liver has emerged as a critical factor promoting the development of non-alcoholic steatohepatitis (NASH) and potentially HCC[3-7], with monounsaturated fatty acids (MUFA) implicated in a pathophysiological role[6,8]. Recent observations also highlighted the accumulation of free cholesterol as an important trigger for the progression from simple steatosis to severe NASH[9-11]. In fact, dietary cholesterol was demonstrated to be a critical factor in the progression of NASH[10,12]. Cholesterol fed to LDLR$^{-/-}$ mice induced a prominent inflammatory response, whereas high fat feeding without cholesterol induced steatosis in the absence of inflammation[13].

The insulin-like growth factor (IGF) 2 mRNA binding protein p62/IMP2-2/insulin-like growth factor 2 mRNA binding protein 2-2 (IGF2BP2-2) is a splice variant of IMP2/IGF2BP2 and was originally described as an autoantigen in an HCC patient[14]. p62 is upregulated in human HCC and its expression is correlated with poor prognosis[15,16]. Only physiological roles have been described for IMP2, which is required for proper nerve cell migration and morphology during development by controlling cytoskeletal remodeling and dynamics (reviewed in[17]). We recently reported that mice with liver-specific overexpression of p62 develop a fatty liver and show increased development towards NASH-induced fibrosis[4,18,19]. This amplification of an inflammatory response was observed in a feeding model that omitted dietary cholesterol. We therefore aimed to investigate the mechanisms involved in the amplification of NASH by p62 in the absence of dietary cholesterol.

A methionine/choline deficient (MCD) diet without supplementation of cholesterol was fed to *p62* transgenic animals. The MCD diet is the most commonly used murine dietary model for acquired NASH, since in contrast to other models, it allows examination of all stages of NASH (*i.e.*, inflammation, oxidative stress, and fibrogenic changes)[20].

Our data implicate p62 as a modulator of endogenous cholesterol synthesis leading to elevated levels of free cholesterol in the liver, ultimately promoting inflammation *via* the activation of nuclear factor kappa B (NF-κB). Moreover, the elevated levels of hepatocellular iron link lipid metabolism to the promotion of inflammatory reactions[21].

MATERIALS AND METHODS

Material

The MCD diet (#960439) and the methionine/choline supplemented control (ctrl) diet (#960441), both containing 45% sucrose and 10% corn oil without cholesterol, were purchased from MP Biomedicals (Heidelberg, Germany). Polymerase chain reaction (PCR) primers were obtained from Eurofins MWG Operon (Ebersberg, Germany). The EvaGreen® qPCR Mix was obtained from Solis BioDyne (Tartu, Estonia). Antibodies and immunofluorescence conditions are detailed in Table 1.

Animal treatment

All animal procedures were performed under the guidelines of the local animal welfare committee (permission No. 34/2010). Mice were kept on a 12-h dark-light cycle under controlled conditions (temperature: 22 ℃ ± 2 ℃; relative humidity: 55% ± 10%) with unrestricted access to food and water until the age of three weeks.

Mice were randomly divided into experimental groups

Table 1 Antibody dilution, unmasking, incubation time, temperature, and immunodetection used for immunofluorescence

Antibody (source)	Unmasking of antigens	Dilution	Incubation	Detection system
NF-κB p65 (Neomarkers, United States)	Citrate buffer pH 6.0, 95 ℃, 10 min, water bath	1:1000	18 h at 4 ℃	IF with Alexa Fluor 546 (Invitrogen, Germany) as secondary antibody

NF-κB: Nuclear factor kappa B; IF: Immunofluorescence.

Table 2 Primer sequences for real-time reverse transcription-polymerase chain reaction

mRNA	Accession No.	Sense primer, 5'→3'	Antisense primer, 5'→3'
18S	NR_003278.1	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
PPARa	NM_001113418.1	CCTTCCCTGTGAACTGACG	CCACAGAGCGCTAAGCTGT
IL-1B	NM_008361.3	GAGAGCCTGTGTTTTCCTCC	GAGTGCTGCCTAATGTCCC
TNF	NM_013693.2	CCATTCCTGAGTTCTGCAAAGG	AGGTAGGAAGGCCTGAGATCTTATC
HMGCR	NM_008255.2	ATCCAGGAGCGAACCAAGAGAG	CAGAAGCCCCAAGCACAAAC
SCD1	NM_009127.4	AGATCTCCAGTTCTTACACGACCAC	CTTTCATTTCAGGACGGATGTCT
CPT1a	NM_013495.2	CTCAGTGGGAGCGACTCTTCA	GGCCTCTGTGGTACACGACAA
NOS2	NM_010927.3	CTCACTGGGACAGCACAGAA	GATGTGGCCTTGTGGTGAA
PTGS/COX2	XM_192868	TGACCCCCAAGGCTCAAATAT	TGAACCCAGGTCCTCGCTTA
FASN	NM_007988.3	GGCTGCTACAAACAGACCAT	CACGGTAGAAAAGGCTCAGT
SREBF2	NM_033218.1	ACCTAGACCTCGCCAAAGGT	CGGATCACATTCCAGGAGA

PPARa: Peroxisome proliferator-activated receptor a; IL-1B: Interleukin 1B; TNF: Tumor necrosis factor; HMGCR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; SCD1: Stearoyl-CoA desaturase 1; CPT1a: Carnitine palmitoyl transferase 1a; NOS2: Nitric oxide synthase 2; PTGS/COX2: Prostaglandin-endoperoxide synthase 2; FASN: Fatty acid synthase; SREBF2: Sterol regulatory element binding transcription factor 2.

Table 3 Scoring system for hepatocellular iron and nuclear factor kappa B-p65 nuclear translocation

Scoring system		Assessed by
Hepatocellular iron		
Score 0	No granules	Prussian blue
Score 1	Zone 1, granules seen at × 40	
Score 2	Granules seen at × 20	
Score 3	Granules seen at × 10	
Score 4	Granules seen at × 10 in zone 1 and 2	

NF-κB: Nuclear factor kappa B; IF: Immunofluorescence.

at the age of 3 wk and fed an MCD diet or an MCD diet supplemented with choline bitartrate (2 g/kg) and DL-methionine (3 g/kg) for two or four weeks; the latter was designated as the ctrl diet[18]. Male and female wild-type or *p62* transgenic mice were used as previously described[19].

Serum parameters

Animals were sacrificed and serum levels were determined at the Zentrallabor des Universitätsklinikums des Saarlandes (Homburg, Germany).

Real-time reverse transcription-polymerase chain reaction

Experiments and quantification were performed as previously described[22]. Primer sequences are listed in Table 2.

Quantification of thiobarbituric acid reactive substances

Products of lipid peroxidation were measured by a fluorimetric assay. Liver tissues (10-20 mg) were homogenized in PBS (Na_2HPO_4 8.0 mmol/L, KH_2PO_4 1.5 mmol/L, NaCl 160 mmol/L in water) containing 1% phosphatase inhibitor cocktail Ⅱ (Sigma-Aldrich, Taufkirchen, Germany), and centrifuged. For protein precipitation, 100 μL lysate was mixed with 200 μL ice cold 10% trichloroacetic acid and after incubation on ice, centrifuged for 10 min at 14000 *g*. The clear supernatant was mixed with an equal volume of thiobarbituric acid (TBA) [0.67% (w/v)], and heated for 15 min at 100 ℃. After cooling the supernatant down to room temperature, the fluorescence intensity of the samples was measured in duplicate in a 96 well plate at $\lambda_{ex/em}$ = 530 nm/572 nm. Thiobarbituric acid reactive substances (TBARS) are expressed as malondialdehyde (MDA) equivalents as μmol per mg liver tissue. An MDA standard was used to create a standard curve, against which unknown samples were plotted.

Fatty acid profile analysis

Snap-frozen liver tissue samples were lyophilized until dry (approximately 10 mg dry weight). Fatty acid extraction and alkaline hydrolysis was performed using the fatty acid methyl ester method and measured with GC-MS as previously published[3,23].

Histology and immunohistochemistry

For histological examination, paraffin-embedded liver tissue specimens were cut and stained with Prussian blue to evaluate iron accumulation. Immunofluorescent staining was performed after unmasking of the sections (Table 1). The sections were immunostained with the appropriate antibody; concentration and incubation time and temperature are listed in Table 1. Staining for unesterified "free" cholesterol was performed on frozen liver sections with filipin, which identifies free cholesterol[11]. Quantification was performed with Image J software on five randomly

Table 4 Liver weight and serum parameters of *p62* transgenic and wild-type mice fed a methionine-choline deficient diet or control diet for two and four weeks

	2 wk				4 wk			
	ctrl		MCD		ctrl		MCD	
	wt	tg	wt	tg	wt	tg	wt	tg
Number of animals	10	10	12	12	10	10	12	12
Relative liver weight (% of body weight)	4.8 ± 0.1	4.6 ± 0.1	3.4 ± 0.1[c]	4.0 ± 0.2[a,c,e]	4.2 ± 01	4.1 ± 0.1	3.4 ± 0.1[c]	3.5 ± 0.2[c,e]
Serum ALT (U/L)	289 ± 83	233 ± 20	418 ± 36[c]	469 ± 73[c,e]	189 ± 38	222 ± 25	235 ± 40	209 ± 46
Serum AST (U/L)	1568 ± 224	1535 ± 162	2404 ± 125[c]	2544 ± 207[c,e]	1845 ± 204	1712 ± 253	2813 ± 286[c]	3057 ± 346[c]
Serum triglycerides (mg/dL)	244 ± 19	219 ± 17	107 ± 5[c]	128 ± 11[c,e]	211 ± 18	203 ± 21	95 ± 7[c]	106 ± 5[c,e]
Serum HDL (mg/dL)	94 ± 6	98 ± 4	24 ± 2[c]	21 ± 4[c,e]	112 ± 8	119 ± 8	15 ± 2[c]	18 ± 2[c,e]
Serum glucose (mg/dL)	234 ± 27	179 ± 15	67 ± 10[c]	59 ± 7[c,e]	193 ± 13	253 ± 36	59 ± 7[c]	40 ± 5[a,c,e]

[a]$P < 0.05$ *vs* wild-type; [c]$P < 0.05$ *vs* control diet; [e]$P < 0.05$ *vs* wild-type on control diet; Values are expressed as the mean ± SEM; tg: Transgenic; wt: Wild-type; ctrl: Control; MCD: Methionine-choline deficient.

selected pictures from each sample. NF-κB-p65 staining was performed as previously published[24]. Counterstaining with either Nuclear fastred for histochemistry or 4',6'-diamidine-2-phenylindol (DAPI) for immunofluorescence (IF) was performed and sections were dehydrated and embedded with Entellan® (#107961, Merck, Germany). As a negative control, sections were incubated without primary antibody.

Three investigators, blinded to all experimental conditions (SMK, RMB, JH), examined the sections for hepatocellular iron and NF-κB-p65 nuclear translocation (Table 3).

Statistical analysis

Data analysis and statistics were performed using Microsoft Office 2010 and OriginPro 8.6 G. The effect of genotype, MCD diet, and their interactions were displayed as mean values ± SEM with 10-12 animals per group. Statistical differences were estimated using the Kruskal-Wallis-ANOVA for nonparametric samples followed by post-hoc-analysis with the Mann-Whitney *U* test. Differences were considered statistically significant when *P* values were less than 0.05.

RESULTS

General effects of dietary manipulation

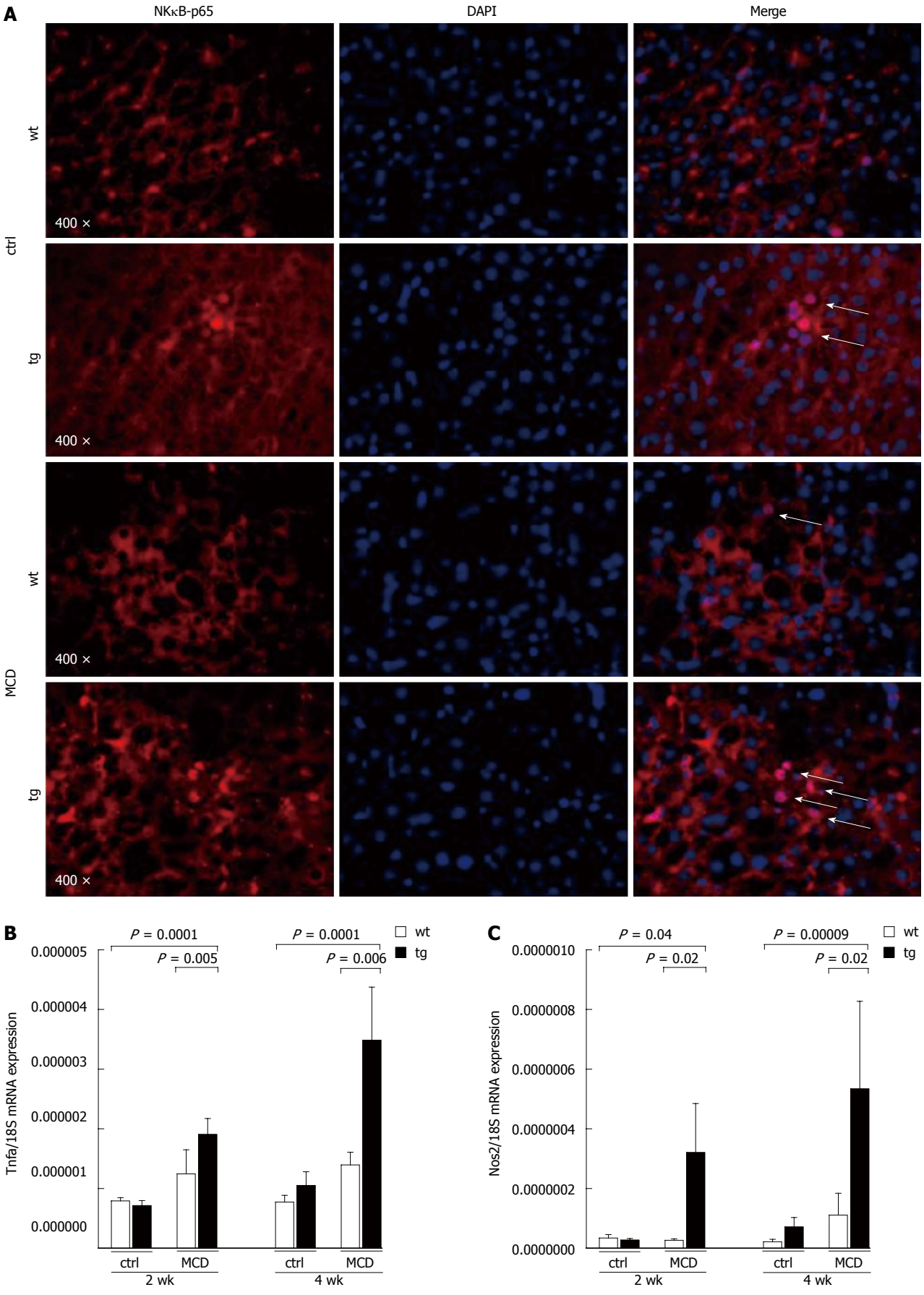
Mice of both genotypes exhibited different characteristics typical of the MCD diet. Specifically, we observed a loss of body mass and relative liver weight through feeding the MCD diet (Table 4). Due to reduced very low density lipoprotein (VLDL) secretion from the liver[25], serum triglycerides and cholesterol were reduced in MCD animals, as were serum glucose levels. *p62* transgenic animals exhibited a further reduction in serum glucose levels as previously reported[18], most likely due to elevated IGF2 production. Elevated aspartate aminotransferase (AST) and alanine transaminase (ALT) levels indicated liver damage induced by the MCD diet, as previously described[26] (Table 4).

p62 amplifies inflammation

Since p62 promotes NASH-induced fibrosis paralleled by increased expression of the chemokine monocyte chemoattractant protein 1 (MCP1)/chemokine ligand 2 (CCl2)[18], our previous data suggested that p62 elicits an increased inflammatory response during NASH. Histological detection of activated NF-κB was assessed by nuclear translocation of its subunit *p65*, and suggested an elevated immune response in *p62* transgenics (Figure 1A). This elevated response was confirmed by an NF-κB-dependent gene expression profile, which showed increased levels of tumor necrosis factor (*TNF*) (Figure 1B), inducible nitric oxide synthase 2 (*Nos2*) (Figure 1C), prostaglandin-endoperoxide synthase 2 (*PTGS/COX2*) (Figure 1D), and interleukin (*IL*) 1B in *p62* transgenic mice (Figure 1E).

p62 alters the fatty acid pattern

Animals of both genotypes developed steatosis on the MCD diet. However, previous histological analyses suggested an amplification of steatosis in *p62* transgenic animals compared to their wild-type littermates[18]. After two weeks, the relative liver weight was significantly increased in transgenics ($P = 0.02$) (Table 4) and therefore consistent with the histological changes. GC-MS analyses revealed significantly higher levels of hepatic fatty acids in *p62* transgenic mice (Figure 2A), whereas serum triglycerides were unchanged (Table 4). The hepatic fatty acid pattern indicated strong alterations in *p62* transgenic animals compared to their wild-type littermates after two weeks on the MCD diet (Table 5). In particular, a more pronounced accumulation of MUFA compared to saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) was observed in transgenic animals (MUFA: 68% increase *vs* SFA: 40% and PUFA: 45%) (Figure 2B). Both the distinct elevation of palmitoleic acid (C16:1) and oleic acid (C18:1) (Figure 2C) indicated increased desaturase activity. Gene expression of the desaturase stearoyl-CoA desaturase (*SCD*) 1, which is responsible for the formation of C16:1 and C18:1 fatty acids, tended to be increased in *p62* transgenic animals, despite a strong downregula-


A
NKκB-p65
DAPI
Merge
ctrl
wt
tg
MCD
wt
tg
400 ×
400 ×
400 ×
400 ×
B
P = 0.0001
P = 0.005
P = 0.0001
P = 0.006
wt
tg
Tnfa/18S mRNA expression
0.000005
0.000004
0.000003
0.000002
0.000001
0.000000
ctrl
MCD
ctrl
MCD
2 wk
4 wk
C
P = 0.04
P = 0.02
P = 0.00009
P = 0.02
wt
tg
Nos2/18S mRNA expression
0.0000010
0.0000008
0.0000006
0.0000004
0.0000002
0.0000000
ctrl
MCD
ctrl
MCD
2 wk
4 wk

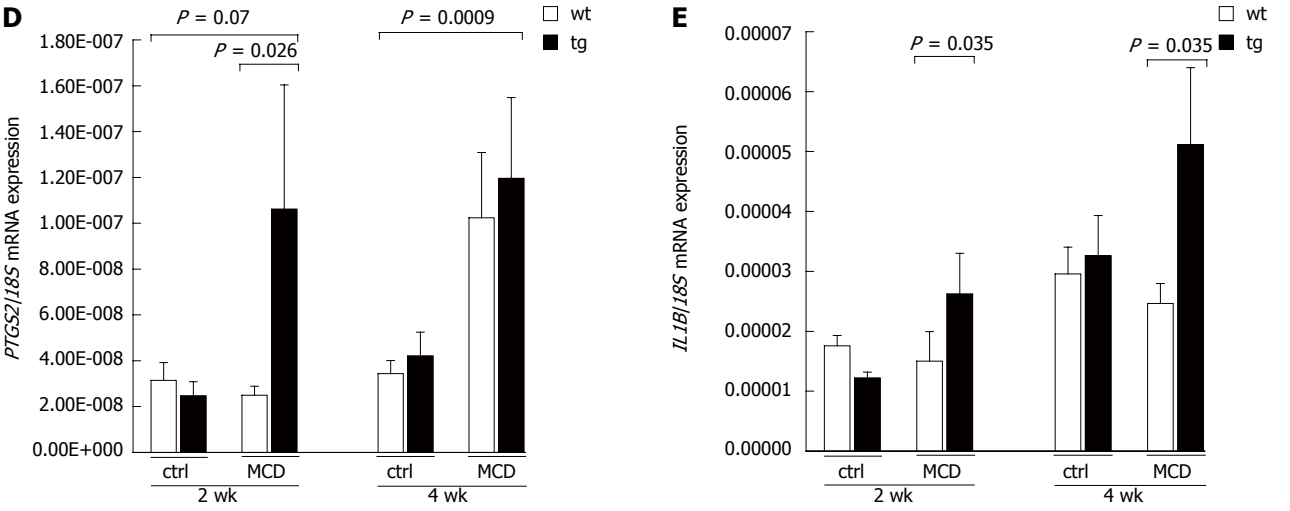


Figure 1 p62 expression amplifies inflammation. A: Immunofluorescent staining with anti-nuclear factor kappa B (NF-κB)-p65 (red, left panel), 4',6-diamidino-2-phenylindole (DAPI) for nuclei (blue, middle panel), and merge (right panel) shows activation of NF-κB after four weeks on the methionine-choline deficient (MCD) diet through *p65* translocation to the nucleus (white arrows) (original magnification: × 400); B-E: Gene expression analysis from quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) of NF-κB target genes with tumor necrosis factor (TNF) (B), inducible nitric oxide synthase 2 (*NOS2*) (C), prostaglandin-endoperoxide synthase 2 (*PTGS/COX2*) (D), and interleukin 1B (*IL-1B*) (E) from whole livers are expressed as a ratio against the housekeeping gene, *18S*. Data are presented as the mean ± SEM (*n* = 10-12). ctrl: Control; wt: Wild type; tg: Transgenie.

tion on the MCD diet (Figure 2D). Expression of other lipogenic and fatty acid catabolism regulators, such as the fatty acid synthase (*FASN*), the lipolysis regulator peroxisome proliferator-activated receptor a (*PPARa*), and the promotor of β-oxidation, carnitine palmitoyl transferase 1a (*CPT1a*), were not altered upon *p62* expression when mice were fed the MCD diet (Figure 2D).

Elevated cholesterol in p62 transgenic animals

Both liver cholesterol as well as serum cholesterol were distinctly elevated in *p62* transgenic mice (Figure 3A and B). Filipin staining for free cholesterol revealed a significant increase in free cholesterol in *p62* transgenic animals on the MCD diet (Figure 3C, D). While the mRNA levels of *HMGCR* were not significantly upregulated (Figure 3E), the expression of the cholesterol metabolism-related transcription factor sterol regulatory element binding transcription factor 2 (*SREBF2*) was significantly increased after four weeks (Figure 3F).

p62 induces hepatocellular iron deposition and lipid peroxidation

Because cholesterol biosynthesis was previously reported to be induced by elevated hepatic iron[27], iron deposition was next evaluated. Both genotypes on the MCD diet had elevated hepatic iron deposition with a more distinct iron accumulation in *p62* transgenic mice (Figure 4A, B). Interestingly, iron deposition was also detected in transgenic, but not in wild-type mice on the ctrl diet (Figure 4B). Since hepatocellular iron is a promoter of oxidative stress, we assessed lipid peroxidation. Accordingly, p62 expression significantly increased lipid peroxidation as analyzed by a TBARS assay on the control diet at two weeks and, on the MCD diet, a respective trend was observed at four weeks (Figure 4C).

DISCUSSION

The fatty acid composition and the accumulation of free cholesterol have been implicated in playing a critical role in the development of NASH since an altered lipidome[3,11,12] as well as free cholesterol, positively correlate with the severity of NASH[10,12,28,29]. Dietary cholesterol may play a role in NAFLD onset[30]. In the current study, we describe an increased production of free cholesterol in mice with liver-specific overexpression of p62 in the absence of dietary cholesterol.

Because elevated activation of NF-κB is observed in *p62* transgenic animals, they are more prone to an inflammatory response in this model of NASH. Accordingly, gene expression of inflammatory mediators such as TNF were elevated in these animals, which has previously been shown for MCP1/CCl2[18]. TNF is strongly correlated with fatty acid metabolism as it negatively regulates the expression of PPARa, leading to decreased catabolism[31]. In human NAFLD, elevated serum TNF levels in patients are a strong indicator for the progression from steatosis to NASH[32].

Surprisingly, we detected a lower apoptosis rate in *p62* transgenic mice when we examined cleaved caspase-3 by immunohistochemistry, counteracting the apoptosis-inducing effect of TNF[33]. Additionally, *p62* transgenic animals did not show an induction of liver damage despite elevated fat and inflammation, which is in contrast to elevated AST and ALT levels in human NASH[34]. The combination of less apoptosis and liver damage confirms the cytoprotective properties of p62[16,19] and may highlight a correlation with the cytoprotective actions of MUFAs[6]. The increase of *IL-1B* mRNA as a result of NF-κB activation in *p62* transgenic animals might further link early lipidomic changes in steatosis with progression to a

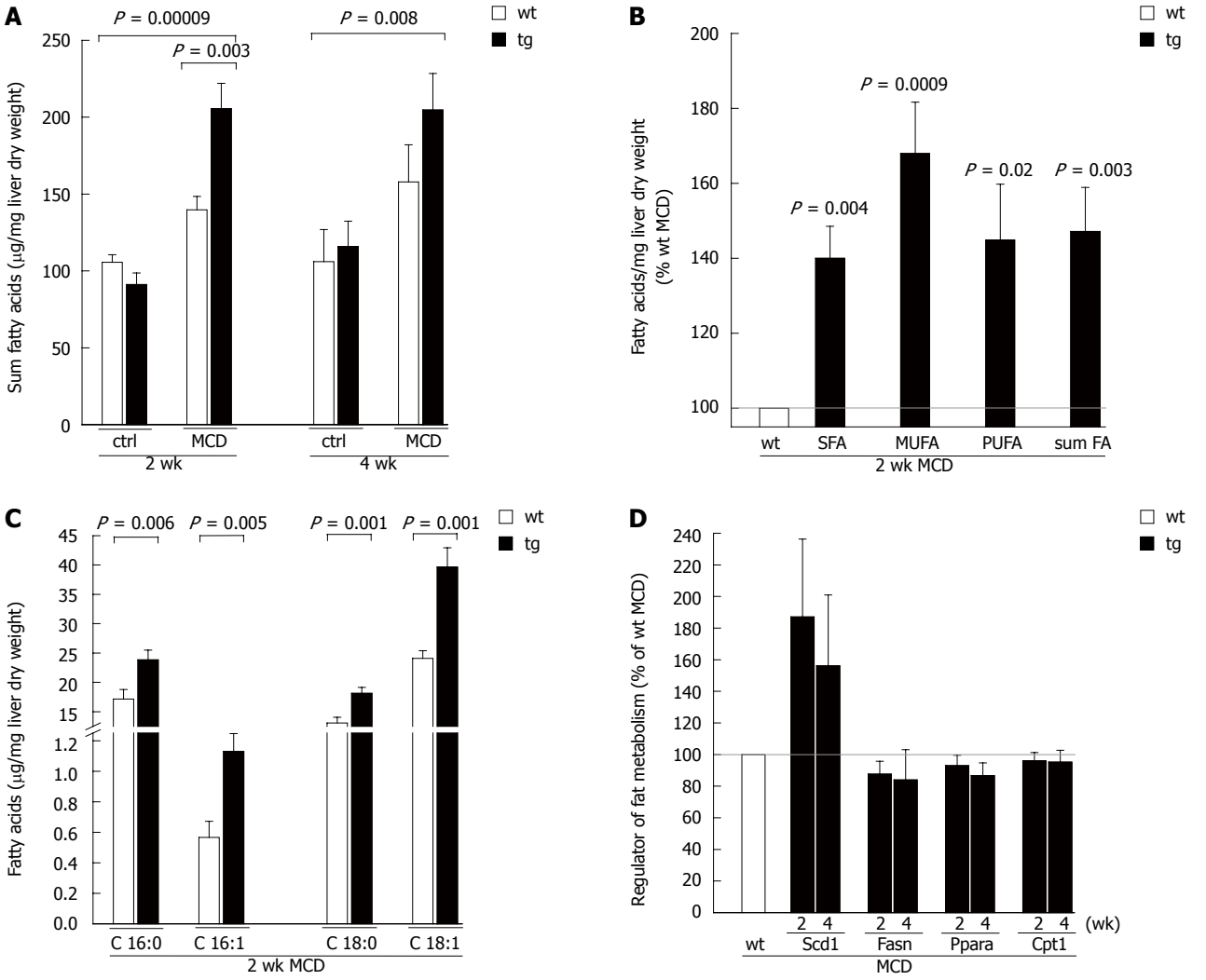


Figure 2 p62 alters the fatty acid pattern. A: Sum of all fatty acids in mice fed the methionine-choline deficient (MCD) or ctrl diet for two and four weeks. Liver tissues were lyophilized, lipids were hydrolyzed, and fatty acids (FA) were analyzed by gas-chromatography mass-spectrometry (GC-MS); B: Sum of the saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and all fatty acids (sum FA) from animals fed the MCD diet for two weeks are presented as the mean ± SEM (n = 9-12). Data are displayed as the percentage of MCD-fed wild-type mice, which were set to 100%, each; C: Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1) are presented as the mean ± SEM (n = 9-12); D: Relative hepatic mRNA expression of stearoyl-CoA desaturase (SCD) 1, fatty acid synthase (FASN), peroxisome proliferator-activated receptor a (PPARa), and carnitine palmitoyl transferase (CPT) 1a from mice fed the MCD diet for two and four weeks were normalized against the housekeeping gene *18S* and are shown as the percentage of MCD-fed wild-type mice, which were set to 100%, each. Data are presented as the mean ± SEM (n = 10-12). tg: Transgenic; wt: Wild-type; ctrl: Control.

strong inflammatory response[35,36].

Interestingly, the amount of MUFA was increased relative to SFA and PUFA in *p62* transgenic animals, indicating that there are alterations in the fatty acid metabolism in both NASH and NASH-related HCC[3,6]. Increased MUFAs are correlated with hypertriglyceridemia and obesity[37], without exogenous ingestion, due to hepatic synthesis. In animal studies, exogenous MUFAs were found to be protective against MCD-induced NASH[8].

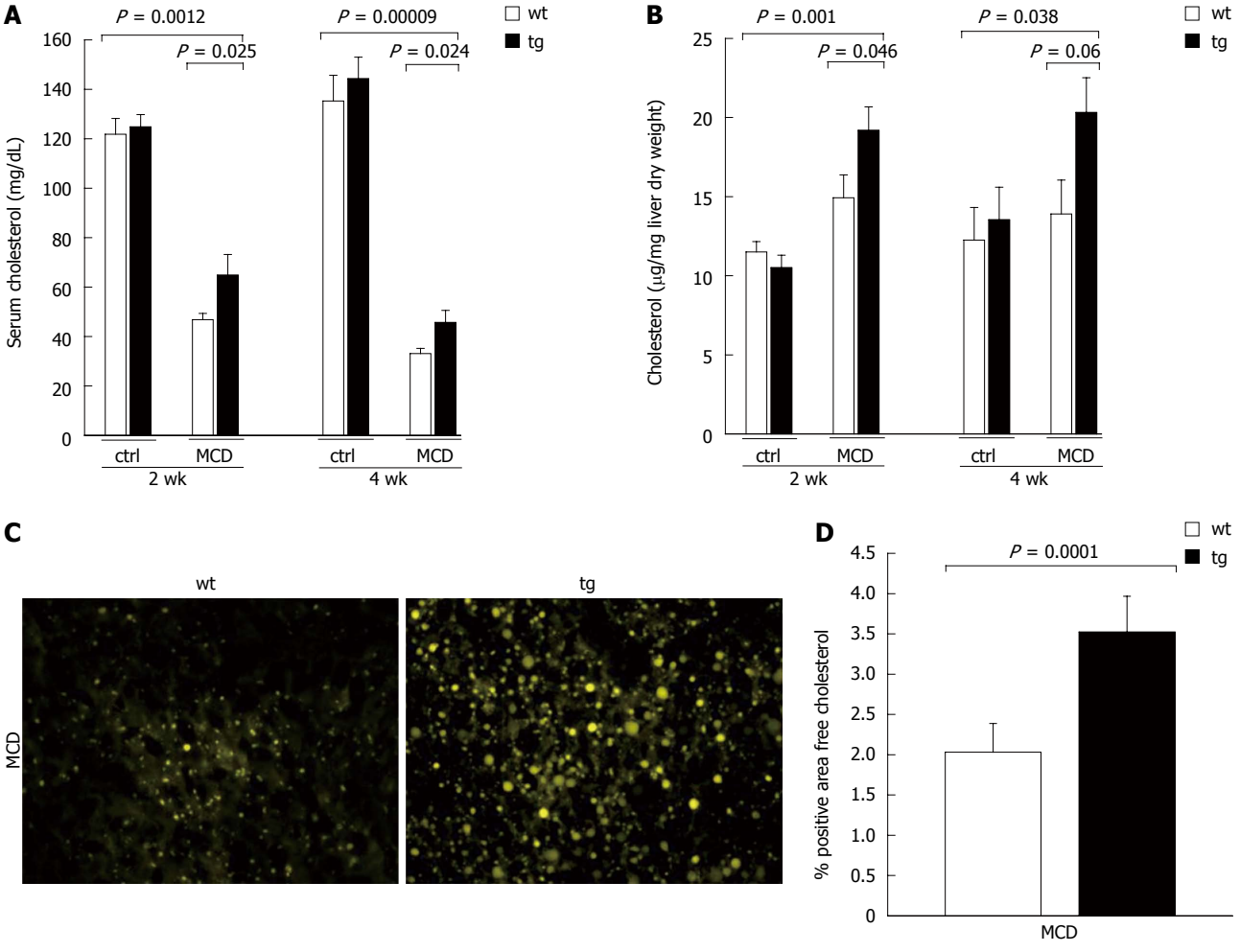
Desaturases represent the rate-limiting enzymes for the production of palmitoleic (C16:1) and oleic acid (C18:1), with SCD1 being the predominant form in the liver[38,39]. In this study, we observed an increase in SCD1 in *p62* transgenic animals, despite a strong downregulation on the MCD diet. In human NASH-related HCC tissues, an upregulation of SCD1 was also found[6]. The expression of FASN was found to be downregulated with the MCD diet without differences among the genotypes, similar to other murine models of steatohepatitis[31,40] and human NASH[28]. We also found no changes for the lipolysis regulators PPARa and CPT1 in *p62* transgenic mice.

p62 transgenic mice fed the MCD diet showed hyperlipidemia with increased serum cholesterol levels. These findings are particularly interesting given that the MCD diet is known for lowered serum triglyceride (TG) levels and, in this particular case, differs from human NASH[41]. To our knowledge, this is the first time that an increase in hepatic total and free cholesterol has been documented in a nutritional mouse model without exogenous cholesterol. Increased dietary cholesterol intake is associated with risk and severity of NAFLD and is paralleled by hepatic free cholesterol accumulation in human as well as in experimental settings[42] and even differentiates steatosis from

Table 5 Gas-chromatography mass-spectrometry fatty acid analyses of mice fed the methionine-choline deficient or control diet for two weeks

Fatty acid	ctrl wt	ctrl tg	*P* value[1]	MCD wt	MCD tg	*P* value[2]	*P* value[3]
14:0	0.25 ± 0.04	0.25 ± 0.05	0.970	0.24 ± 0.04	0.43 ± 0.04	0.003	0.005
15:0	0.01 ± 0.003	0.01 ± 0.003	0.113	0.02 ± 0.01	0.05 ± 0.01	0.035	0.002
16:0	17.23 ± 0.79	16.08 ± 1.43	0.623	17.16 ± 1.63	23.85 ± 1.65	0.006	0.0033
16:1	1.69 ± 0.29	1.51 ± 0.30	0.570	0.57 ± 0.10	1.13 ± 0.12	0.005	0.138
17:0	0.11 ± 0.01	0.06 ± 0.01	0.021	0.23 ± 0.03	0.34 ± 0.03	0.010	0.0001
17:1	0.02 ± 0.01	0.01 ± 0.01	0.046	0.003 ± 0.003	0.01 ± 0.01	0.514	0.117
18:0	12.64 ± 0.57	9.41 ± 0.81	0.006	13.08 ± 0.97	18.15 ± 1.00	0.0014	0.0009
18:1	20.13 ± 2.51	17.98 ± 2.07	0.678	24.11 ± 1.28	39.66 ± 3.27	0.001	0.0009
18:2	17.88 ± 0.87	16.42 ± 1.60	0.241	23.90 ± 2.15	36.28 ± 6.34	0.040	0.004
18:3	0.23 ± 0.05	0.27 ± 0.10	0.571	1.50 ± 0.28	2.99 ± 0.46	0.003	0.00009
20:0	0.22 ± 0.05	0.10 ± 0.03	0.045	0.14 ± 0.03	0.33 ± 0.05	0.0007	0.138
20:1	0.40 ± 0.03	0.30 ± 0.04	0.045	0.81 ± 0.14	1.99 ± 0.27	0.002	0.00009
20:2	0.45 ± 0.04	0.53 ± 0.06	0.385	1.67 ± 0.17	2.94 ± 0.25	0.0006	0.00009
20:3	1.09 ± 0.09	0.89 ± 0.14	0.186	2.87 ± 0.37	5.15 ± 0.44	0.0006	0.00009
20:4	13.69 ± 0.58	10.37 ± 0.80	0.011	16.49 ± 1.15	23.20 ± 1.81	0.006	0.00015
22:0	0.59 ± 0.11	0.29 ± 0.07	0.031	0.31 ± 0.03	0.50 ± 0.03	0.0007	0.921
22:1	0.01 ± 0.003	0.003 ± 0.003	0.387	0.01 ± 0.01	0.04 ± 0.01	0.091	0.129
22:4	0.57 ± 0.05	0.51 ± 0.08	0.273	4.24 ± 0.48	6.25 ± 0.56	0.0036	0.00009
22:6	4.72 ± 0.28	3.82 ± 0.29	0.054	12.08 ± 0.90	14.06 ± 1.05	0.214	0.00009
23:0	0.08 ± 0.01	0.04 ± 0.004	0.0003	0.08 ± 0.01	0.10 ± 0.01	0.194	0.223
24:0	0.42 ± 0.02	0.31 ± 0.02	0.003	0.45 ± 0.02	0.63 ± 0.05	0.0013	0.0009

[1]*P* value of the comparison between ctrl wt and ctrl tg; [2]*P* value of the comparison between MCD wt and MCD tg; [3]*P* value of the comparison between ctrl wt and MCD tg; Values (μg/mg dry liver tissue) are expressed as the mean ± SEM. Liver tissues were lyophilized and analyzed by gas-chromatography mass-spectrometry. tg: Transgenic; wt: Wild-type; ctrl: Control; MCD: Methionine-choline deficient.



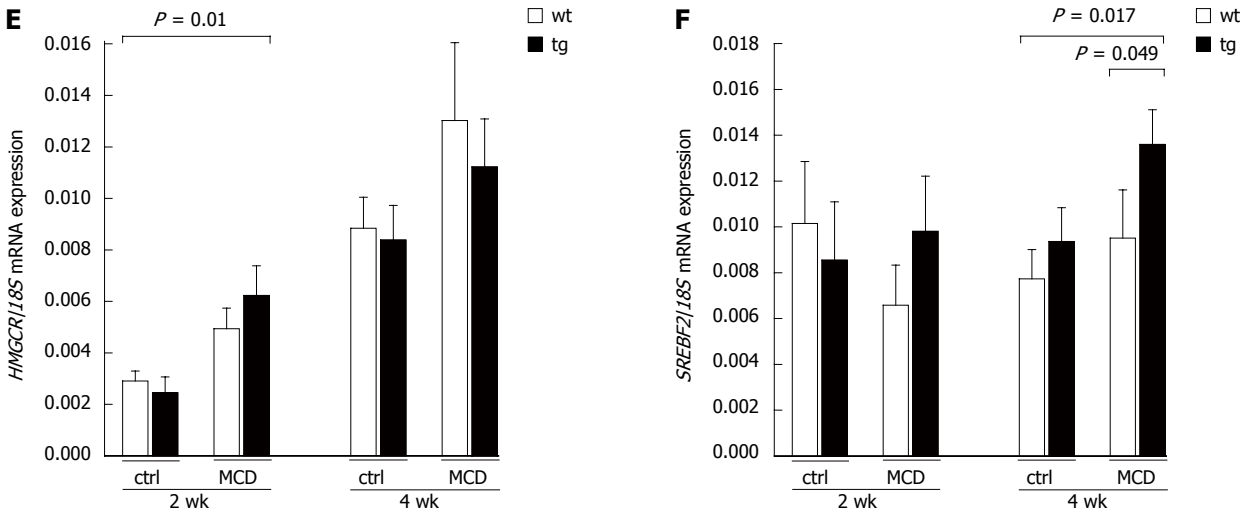
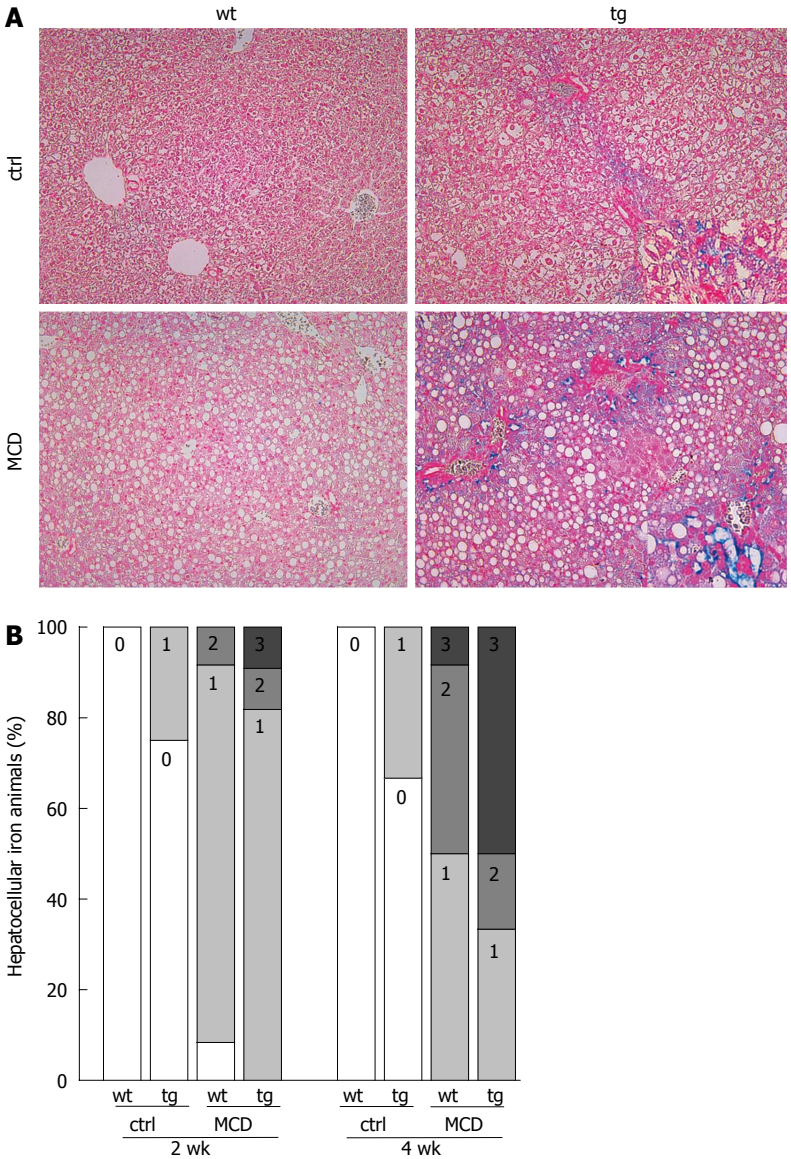


Figure 3 p62 expression elevates serum and liver cholesterol. A, B: Hepatic (A) and serum (B) cholesterol concentrations in mice fed the respective diet for two or four weeks; C, D: Representative cryosections stained with Filipin for hepatic free cholesterol in mice fed the methionine-choline deficient (MCD) diet for four weeks (original magnification: × 400) (C) with corresponding quantification (D) (mean out of five randomly picked sections on the slide); E, F: Relative hepatic mRNA expression of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase or *HMGCR*) (E) and sterol regulatory element binding transcription factor 2 (*SREBF2*) (F) are shown as a ratio against the housekeeping gene, *18S*. Data are presented as the mean ± SEM (n = 10-12). tg: Transgenic; wt: Wild-type; ctrl: Control.



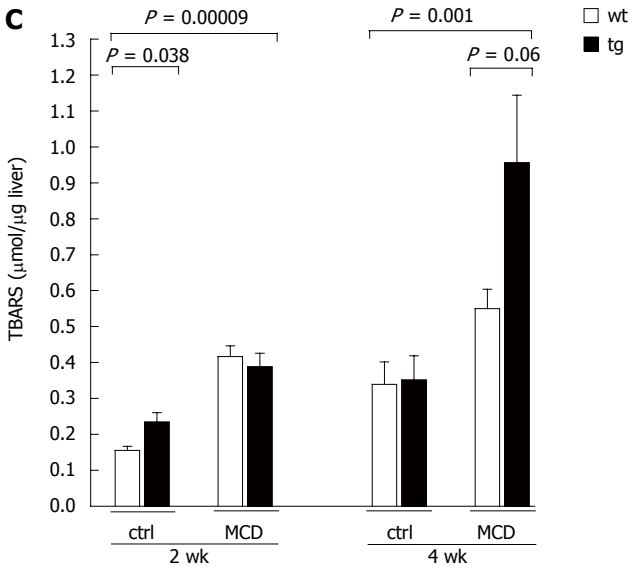


Figure 4 p62 expression leads to increased iron accumulation and reactive oxygen species production. A, B: Representative paraffin-embedded liver sections stained with Prussian blue for iron accumulation from animals fed the respective diet for four weeks (original magnification × 200 and × 500 for inserts) (A) with the corresponding hepatocellular iron score (B) for all time points (for scoring, see supplement S1); C: Hepatic thiobarbituric acid reactive substances (TBARS) were measured to indicate lipid peroxidation and are presented as the mean ± SEM (*n* = 10-12). tg: Transgenic; wt: Wild-type; ctrl: Control; MCD: Methionine and choline deficient.

NASH[43]. Besides dietary cholesterol intake, cellular cholesterol accumulation may result from disturbed cholesterol homeostasis[42]. Here, we observed enhanced expression of SREBF2, but no distinct effect on the expression of the rate-limiting enzyme HMGCR. While a positive correlation between the severity of NASH with the expression of these genes was found[29], others reported no correlation between SREBF2 and hepatic cholesterol despite elevated HMGCR[27]. Interestingly, however, cholesterol biosynthesis was found to be positively correlated with iron accumulation: when additional iron was given, it led to deposition of free cholesterol and an upregulation of cholesterol biosynthesis[27]. Furthermore, hepatocellular iron deposition was reported to be elevated in human NASH patients[44] and NASH-related HCC patients[45]. Variations in hepatic iron levels can directly lead to a modulation of lipogenesis and lipid storage and secretion, as iron is an integral part of several lipid metabolism related enzymes[46]. In this context, SCD1 activity has been shown to be iron-dependent, as the protein contains iron as a cofactor[47]. Accordingly, the iron accumulation in *p62* transgenic mice might elevate SCD1 activity.

Enhanced iron accumulation is also related to enhanced lipid peroxidation[44] since iron is known to catalyze the production of reactive oxygen species, which can then initiate cellular damage and lipid peroxidation[48]. In fact, reactive oxygen species have been suggested as critical contributors to the second hit required for disease onset[49]. Elevated production of reactive oxygen species in *p62* transgenics on the ctrl diet, which correlates with the iron accumulation in these animals, might predispose them towards the development of NASH.

Taken together, this study reveals that liver-specific overexpression of p62 leads to an amplified progression of NAFLD towards NASH through increased production of hepatic free cholesterol driving the inflammatory response in liver disease.

ACKNOWLEDGMENTS

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COMMENTS

Background

In industrialized countries, non-alcoholic fatty liver disease (NAFLD) represents the most frequent chronic liver disease and is a potential risk factor for the development of hepatocellular carcinoma (HCC), the most common primary liver cancer. HCC, an aggressive cancer with high mortality, is difficult to detect and treat. The insulin-like growth factor (IGF) 2 mRNA binding protein p62 was originally discovered in an HCC patient. p62 induces fatty liver and promotes non-alcoholic steatohepatitis (NASH)-induced fibrosis.

Research frontiers

Dietary cholesterol represents a critical factor in the development of NASH from hepatic steatosis. In this context, the accumulation of free cholesterol was recently highlighted as important trigger for the progression from simple steatosis to severe NASH. Recently, the role of free cholesterol and iron accumulation in NASH progression have been actively researched. Whether and how elevated free cholesterol can accumulate independently of dietary cholesterol is a current topic of strong interest.

Innovations and breakthroughs

The authors show for the first time that free cholesterol can accumulate and promote NAFLD in the absence of dietary cholesterol. The IGF2 mRNA binding protein p62 facilitates increased levels of both free cholesterol and hepatic iron. Furthermore, hepatic iron accumulation was associated with lipid peroxidation. In summary, this study shows that p62 drives the progression of NASH by increasing hepatic free cholesterol.

Applications

The understanding of how p62 promotes NASH progression and further characterization of the role of specific lipid changes will increase knowledge about

NASH pathogenesis and might therefore aid in the development of preventive strategies against NASH and NASH-associated HCC. This might also lead to new therapeutic options in NASH treatment.

Terminology

Free cholesterol: cholesterol not esterified with a fatty acid.

Peer review

The data show that p62/IGF2BP2-2 drives the progression of NASH by increasing hepatic free cholesterol. This study is well executed and relevant to clinicians and scientists studying NASH and NAFLD.

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ORIGINAL ARTICLE

Senescent human hepatocytes express a unique secretory phenotype and promote macrophage migration

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Abstract

AIM: To develop a model of stress-induced senescence to study the hepatocyte senescence associated secretory phenotype (SASP).

METHODS: Hydrogen peroxide treatment was used to induce senescence in the human HepG2 hepatocyte cell line. Senescence was confirmed by cytochemical staining for a panel of markers including Ki67, p21, heterochromatin protein 1β, and senescence-associated-β-galactosidase activity. Senescent hepatocytes were characterised by gene expression arrays and quantitative polymerase chain reaction (qPCR), and conditioned media was used in proteomic analyses, a human chemokine protein array, and cell migration assays to characterise the composition and function of the hepatocyte SASP.

RESULTS: Senescent hepatocytes induced classical markers of senescence (p21, heterochromatin protein 1β, and senescence-associated-β-galactosidase activity); and downregulated the proliferation marker, Ki67. Hepatocyte senescence induced a 4.6-fold increase in total secreted protein ($P = 0.06$) without major alterations in the protein profile. Senescence-induced genes were identified by microarray (Benjamini Hochberg-corrected $P < 0.05$); and, consistent with the increase in secreted protein, gene ontology analysis revealed a significant enrichment of secreted proteins among inducible genes. The hepatocyte SASP included characteristic factors such as interleukin (IL)-8 and IL-6, as well as novel components such as SAA4, IL-32 and Fibrinogen, which were validated by qPCR and/or chemokine protein array. Senescent hepatocyte-conditioned medium elicited migration of inflammatory (granulocyte-macrophage colony stimulating factor, GM-CSF-derived), but not non-inflammatory (CSF-1-

derived) human macrophages (P = 0.022), which could contribute to a pro-inflammatory microenvironment *in vivo*, or facilitate the clearance of senescent cells.

CONCLUSION: Our novel model of hepatocyte senescence provides insights into mechanisms by which senescent hepatocytes may promote chronic liver disease pathogenesis.

Key words: Cell aging; Chemokines; Hepatocytes; Inflammation; Liver diseases; Macrophages

Core tip: Hepatocyte senescence is observed in all chronic liver diseases of hepatocellular origin, even at early stages of disease progression. Although widely studied in cancer biology, the role of cellular senescence in the pathogenesis of inflammatory diseases is not known. We developed a novel model of stress-induced hepatocyte senescence and used it to demonstrate that senescent human hepatocytes adopt a hyper-secretory phenotype, which is likely to condition their microenvironment and contribute to disease pathogenesis. We used microarray and proteomic analysis to characterise senescent hepatocytes and identify candidate mediators; and confirmed the functional relevance of senescence-associated secretory phenotype by demonstrating that conditioned media from senescent hepatocytes elicits inflammatory macrophage migration.

Irvine KM, Skoien R, Bokil NJ, Melino M, Thomas GP, Loo D, Gabrielli B, Hill MM, Sweet MJ, Clouston AD, Powell EE. Senescent human hepatocytes express a unique secretory phenotype and promote macrophage migration. *World J Gastroenterol* 2014; 20(47): 17851-17862 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17851.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17851

INTRODUCTION

In chronic liver diseases such as viral infection and non-alcoholic fatty liver disease (NAFLD), hepatocyte regenerative capacity is impaired due to increased cell turnover and injury by DNA damage or oxidative stress[1,2]. Growth-arrested hepatocytes may evolve to a senescent state, characterized by cell cycle arrest and resistance to growth factor stimulation, despite continued metabolic activity. Senescent cells in liver sections have been detected using various methods, including histochemical staining for β-galactosidase activity (an enzymatic marker associated with replicative senescence) or positive staining for the cyclin-dependent kinase inhibitor, p21, in hepatocyte nuclei. Other cytochemical techniques assess telomere shortening or the formation of senescence-associated heterochromatin foci (SAHF). Using one or more of these techniques, several groups have reported the presence of increased numbers of senescent hepatocytes in chronic hepatitis and cirrhosis[3-5]. Senescent hepatocytes were preferentially located in periportal or periseptal areas[3,6,7], particularly adjacent to areas with prominent mononuclear cell infiltration[8] and bile ductular reaction[9].

Senescence arrests the growth of cells at risk of neoplastic transformation, and it has most frequently been studied in the context of tumour suppression[10]. However, the role of senescence in tumorigenesis is complex, since factors secreted by senescent cells can also promote malignant phenotypes in neighbouring cells, such as proliferation and invasion[11]. The contribution of senescence to non-cancer pathologies has rarely been studied, although it is recognised that senescent cells can have deleterious effects on the tissue microenvironment. Accumulation of senescent cells in chronic liver diseases may contribute to ongoing injury, altered tissue repair and fibrogenesis[11,12]. Recent studies in human alcoholic liver disease and NAFLD demonstrated that, in addition to a strong association between hepatocyte p21 expression and fibrosis stage, there was an independent relationship between the proportion of senescent hepatocytes and an adverse clinical outcome (including hepatocellular cancer, liver transplantation and liver-related death)[13,14]. The mechanistic basis for the pathogenic effects of senescence in chronic liver disease remains unclear, although it is recognized that senescent cells can adversely affect their microenvironment *via* the adoption of specific "secretory" phenotypes (including cytokines, chemokines, growth factors and proteases), which typically have a pro-inflammatory effect on surrounding cells[11]. The senescence-associated secretory phenotype (SASP)[15] has been extensively characterised in other cells, most notably fibroblasts. It is known that different cell types express distinct but overlapping SASPs in response to different triggers of senescence[15], but the SASP of senescent hepatocytes has not been investigated.

The aim of this study was to develop an *in vitro* model to investigate senescence-induced changes in hepatocyte gene expression and secretory profile. The chemokine profile and chemotactic capacity of cultured senescent hepatocytes was specifically examined, in view of their location in diseased tissue adjacent to areas with prominent mononuclear cell infiltration,

MATERIALS AND METHODS

Cell culture and preparation of senescent cell conditioned media

The human hepatoma-derived cell line, HepG2, was purchased from American Type Culture Collection. Unless otherwise indicated, cells were cultured in "complete medium" comprising Dulbecco's modified eagle's medium supplemented with 10% foetal bovine serum (FBS, Invitrogen), 100 U/mL penicillin/100 μg/mL streptomycin/2 mmol GlutaMAX/20 μmol non-essential amino acids, and maintained at 37 ℃/5% CO_2. For dose response assays, HepG2 cells were cultured for 48 h in the presence

of 150-600 μmol/L hydrogen peroxide (H_2O_2, Sigma). Cell viability and metabolic activity was determined using the CellTiter96® Assay (Promega). The degree of cell lysis was determined by assaying for lactate dehydrogenase in culture medium (Sigma). Fully lysed HepG2 cells (treated with 0.25% Triton-X 100) were used as a positive control. To induce senescence, HepG2 cells were seeded at 2×10^5/well in 6 well plates and treated with a single dose of H_2O_2 (300 μmol/L) each day for the first 2 d to induce replicative arrest. Senescence was assessed following culture for a further 7 d (without H_2O_2 treatment), with the media refreshed every 3 d. Due to the proliferative disadvantage of non-senescent cells, control cultures were seeded at 5×10^4 cells/well and cultured in parallel to senescent cultures for 7 d. Cells for immunostaining were seeded on glass coverslips and treated identically. To facilitate study of conditioned media, in some experiments cells were subsequently cultured for 24 h in serum free medium prior to harvest of cells and conditioned medium on day (D) 10. Conditioned culture media was clarified by centrifugation at 600 g, filtered (0.22 μm micropore filters; Corning) and stored in single-use aliquots at -80 ℃. The protein concentration of conditioned culture media was quantified by Bradford Assay (Bio-Rad). To generate human monocyte-derived macrophages (HMDM), $CD14^+$ monocytes were isolated from human buffy coats, obtained from the Australian Red Cross Blood Service. Ficoll-Paque Plus (GE Healthcare) density gradient separation was used to isolate Peripheral Blood Mononuclear Cells followed by positive selection of $CD14^+$ monocytes (Miltenyi Biotech). Isolated $CD14^+$ monocytes (> 90% purity by flow cytometry) were cultured for 7 d in Iscove's Modified Dulbecco's Medium with 10% heat inactivated FBS/50 U/mL Penicillin/50 μg/mL Streptomycin in the presence of human macrophage colony stimulating factor (M-CSF) (1×10^4 U/mL) or granulocyte macrophage colony stimulating factor (GM-CSF) (10 ng/mL) to generate HMDM.

Assays for cellular senescence

Replicative arrest was assessed by $p21^{WAF1/Cip1}$ (EA10, Invitrogen) and Ki-67 staining (SP6, Cell Marque), with DAPI counter-staining for cell nuclei. Senescence was determined by staining for HP1β (1MOD-1A9, Millipore) and senescence-associated β-galactosidase (SA-β-Gal) activity (Sigma). Cells in a single 400 × high power field (HPF) were quantified by counting the number of positive cells as a proportion of total DAPI-stained cells (10 × HPF/treatment). Results were expressed as the proportion of positively-stained nuclei in treated and untreated cell cultures. All experiments were performed three times and results pooled for analysis.

Polyacrylamide gel electrophoresis and mass spectrometry

Standard polyacrylamide gel electrophoresis (PAGE) was performed with the NuPAGE system (10% Bis-Tris gel, MES-SDS running buffer). Two dimensional (2D) gel electrophoresis, in-gel digest and protein identification by tandem mass spectrometry were performed as previously described[16]. To focus on small molecular weight proteins, 12% SDS-PAGE and pH3-11 non-linear IPG gradients were used. Spectrum Mill software (Agilent Technologies) was used for database searching, using the SwissProt database (species *Homo sapiens*, with carbamidomethylated cysteine as fixed modification and oxidized methionine as variable), a maximum missed cleavage of 2, precursor mass tolerance of ± 20 and product mass tolerance of ± 50. Results were filtered by protein score of > 11.0, peptide score of > 10, and % scored peak intensity of > 60.

Microarray analysis of untreated and senescent HepG2 cells

Total RNA was extracted from untreated HepG2 cells cultured for 2 d, as well as control and senescent cells cultured for 10 d (including 24 h in serum free medium), using TRI reagent (Sigma). RNA quality was assessed with an Agilent 2100 BioAnalyser and only samples with a RNA integrity number above 8.0 were included. cRNA was generated from 500 ng total RNA using the Illumina TotalPrep cRNA Amplification Kit (Applied Biosciences) and hybridised to Human HT-12_V3 Expression BeadChips (Illumina). Array data were processed using Illumina GenomeStudio software and imported into Genespring (Agilent) for analysis. Each array was normalised to the 50th percentile probe expression (per array normalisation), and each probe was normalised to its average expression in control, untreated HepG2 cells at day 10. Data were filtered to remove probes that did not reach an Illumina detection score of 1 in at least one sample. A senescence-associated gene expression signature was identified by selecting probes that were differentially expressed between control and senescent cells (D10, ⩾ 1.5 ×, $P < 0.05$, *t*-test with Benjamini Hochberg multiple testing correction), and subtracting probes that were differentially expressed between untreated HepG2 cells at baseline (D2) and D10 (⩾ 1.5 ×, Benjamini Hochberg-corrected $P < 0.05$). Gene ontology (GO) analysis was performed using DAVID[17] with the Illumina HT12 genome as background. Significantly enriched ($P < 0.05$) GO terms, KEGG pathways and sequence features were hand-curated to remove redundant terms.

Real-time polymerase chain reaction

RNA was reverse-transcribed to cDNA using SuperScriptIII Reverse Transcriptase (Invitrogen). Quantitative real-time PCR (qPCR) for genes of interest was performed using SYBR green (ABI) on an HT9000 cycler with default cycle settings. Relative expression was analysed using *HPRT* as housekeeping gene and the delta-Ct method. Primer sequences used in this study were: HPRT, Forward: TCAGGCAGTATAATCCAAAGATGGT; HPRT, Reverse: AGTCTGGCTTATATCCAACACTTCG; IL6, Forward: ATG CAA TAA CCA CCC CTG AC; IL6, Reverse: AAA GCT GCG CAG AAT GAG AT; SAA4,

Forward: CCA AAG CCA GCA GAG GTA CCA AC; SAA4, Reverse: ACG AAC GCC AGC TTT CAC TGG; ECADH, Forward: ATT GCA AAT TCC TGC CAT TC; ECADH, Reverse: GCT GGC TCA AGT CAA AGT CC; IL32, Forward: AGA CAG TGG CGG CTT ATT ATG AG; IL32, Reverse: GCA CCG TAA TCC ATC TCT TTC TTT; IL8, Forward/Reverse: proprietary (Applied Biosystems).

Human chemokine array and macrophage migration assays using conditioned media

The human chemokine antibody array (RD Systems, ARY017) was performed according to the manufacturers' instructions. Macrophage migration was assessed using the xCELLigence (RTCA DP) system (ACEA Biosciences) in 16 well CIM-Plates. The upper chamber of each well of the plate was coated with 0.1% Fibronectin from human plasma (Sigma-Aldrich). The lower chamber was filled with 160 μL control or senescent conditioned media (CM). Thirty microlitres of complete media was added to the top chamber and the set-up was allowed to equilibrate at 37 ℃ for 30 min. 1×10^5 HMDM resuspended in complete media without differentiation factors were added to the top chamber. Impedance readings were collected every 5 min over a 24 h period. xCELLigenece assays were conducted using CM from 4 independent senescence cultures with M-CSF- and GM-CSF-derived HMDM from 2 independent blood donors. Transwell assays were conducted using 24 well transwell culture plates (Corning) with control or senescent conditioned medium in the lower chamber. After 24 h incubation, cells were fixed in paraformaldehyde, and stained with DAPI. Five random images per transwell membrane were captured and nuclei were counted, blinded as to sample identity. Transwell assays were conducted using CM from 4 independent senescence cultures with M-CSF- and GM-CSF-derived HMDM from 1 blood donor.

Statistical analysis

Where not elsewhere described, statistical analyses were conducted in GraphPad Prism, using the Mann-Whitney *U* test to compare between treatment conditions. For independent biological replicates data were expressed as mean ± SE where $n > 3$, or mean ± range where $n = 2$. For technical replicates data were expressed as mean ± SD.

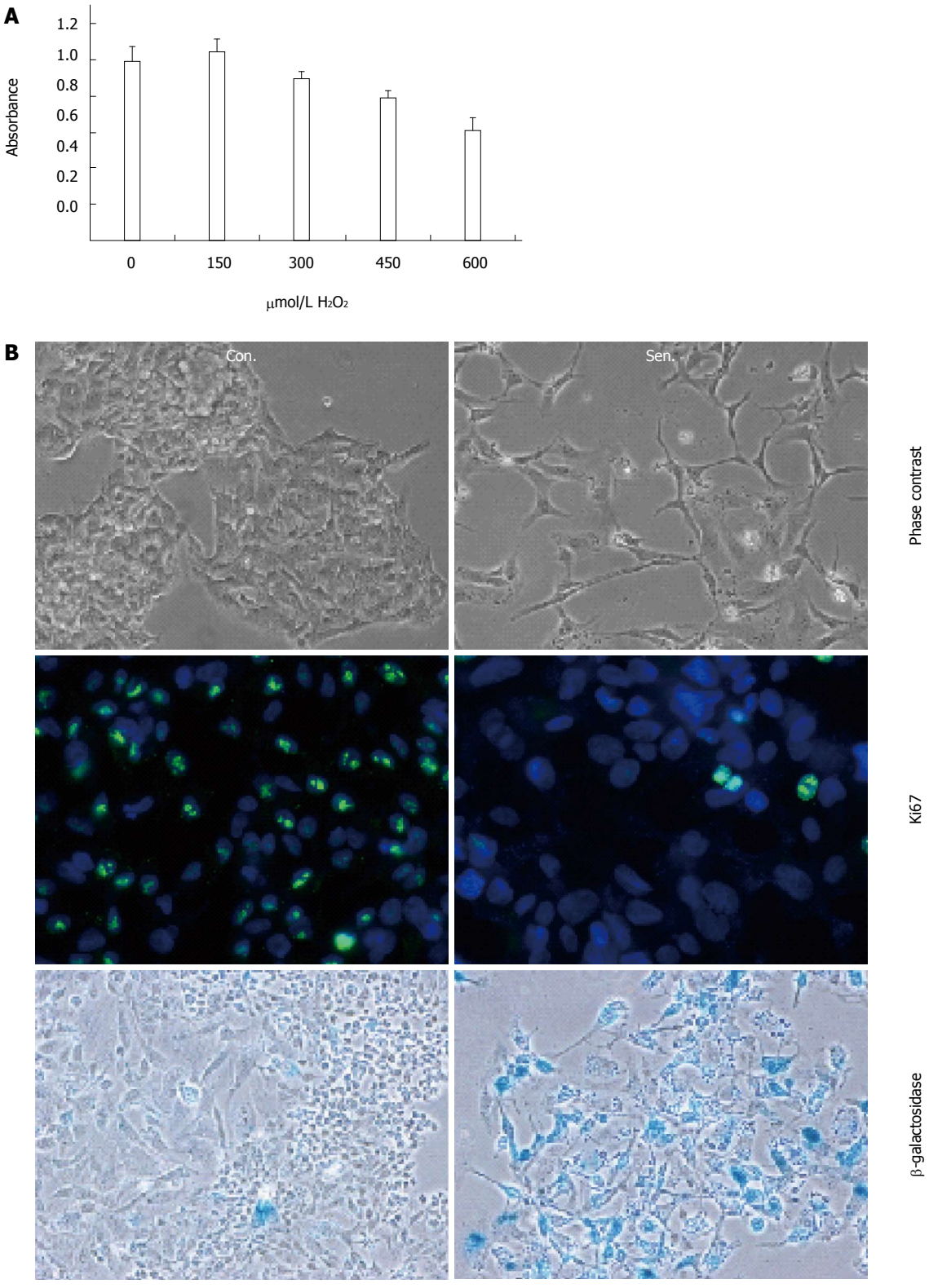
RESULTS

Oxidative stress causes replicative arrest and senescence in HepG2 cells

We sought to establish an *in vitro* model of oxidative stress-induced senescence in the human hepatocyte cell line, HepG2 by exposure to an acute (48 h), sub-lethal dose of H_2O_2, an oxidant produced in normal cellular aerobic metabolism and inflammation. H_2O_2 treatment was previously reported to induce premature senescence in fibroblasts, and has been used as a model of aging[18]. We first performed a H_2O_2 dose response to select a dose that did not affect hepatocyte viability. Treatment with 300 μmol/L H_2O_2 for 48 h did not affect HepG2 metabolic activity (Figure 1A), or survival (lactate dehydrogenase release into culture medium was 8% ± 0.1% and 8.5% ± 0.1% of the lysed cell control for untreated and H_2O_2-treated HepG2 cells respectively). Following treatment, cells were cultured for a further 7 d in the absence of H_2O_2, to allow time for the SASP to develop[11], and 1 d in serum free media (to minimise interference of serum proteins in conditioned media studies). Cells were thus routinely analysed on D10 of the experimental protocol. H_2O_2-treated HepG2 cells adopted a flattened, elongated morphology, typical of senescent cells (Figure 1B). Senescence was confirmed by reduced expression of the proliferation-associated protein Ki67, together with increases in β-galactosidase activity, expression of the cell cycle inhibitor p21, and appearance of nuclear heterochromatin protein-1-beta (HP1β) positive nuclear foci (Figure 1C, D, $P < 0.01$). The involvement of both p21 and SAHF suggests the involvement of both the p53- and p16/Rb-dependent senescence pathways in hepatocytes, consistent with reports of p16/p21 co-expression in senescent liver cells *in vivo*[19].

Senescent hepatocyte secretory profile

One of the cardinal features associated with cellular senescence is an increase in secretory activity[15]. Senescent hepatocytes secreted more protein than control cells on a per cell basis (mean 4.6-fold increase per cell, $P = 0.06$). SDS PAGE analysis of proteins secreted by an equivalent number of control and senescent cells illustrated this increase in protein production, and suggested a similar repertoire of secreted proteins in senescent and control cells (Figure 2A). Analysis of an equivalent amount of protein from control and senescent CM by 2D PAGE further demonstrated that the gross hepatocyte secretory profile in control and senescent hepatocyte conditioned medium was similar, suggesting a generalised upregulation of normal hepatocyte secretory products (Figure 2B, C). Senescent hepatocytes may even express a more limited repertoire of proteins than non-senescent cells, at least for abundant proteins visible by coomassie staining (compare Figure 2B, C). One region of the 2D gel (indicated in Figure 2) that was clearly different between control and senescent CM was excised and subjected to mass spectrometry. 14 proteins in this region were identified by ⩾ 2 unique peptides in senescent CM, compared to 31 in control CM. The most abundant proteins in senescent CM were alpha-fetoprotein and albumin (21/19 spectra respectively, compared to 8/6 spectra in control CM). Complement C3 was the only protein uniquely detected in senescent CM. Since few differences between control and senescent secretory profiles were evident in our preliminary proteomics analysis, and because many SASP factors (such as cytokines and chemokines) may be expressed at low levels, we adopted a transcriptomic approach to further characterise senescent hepatocytes.


A
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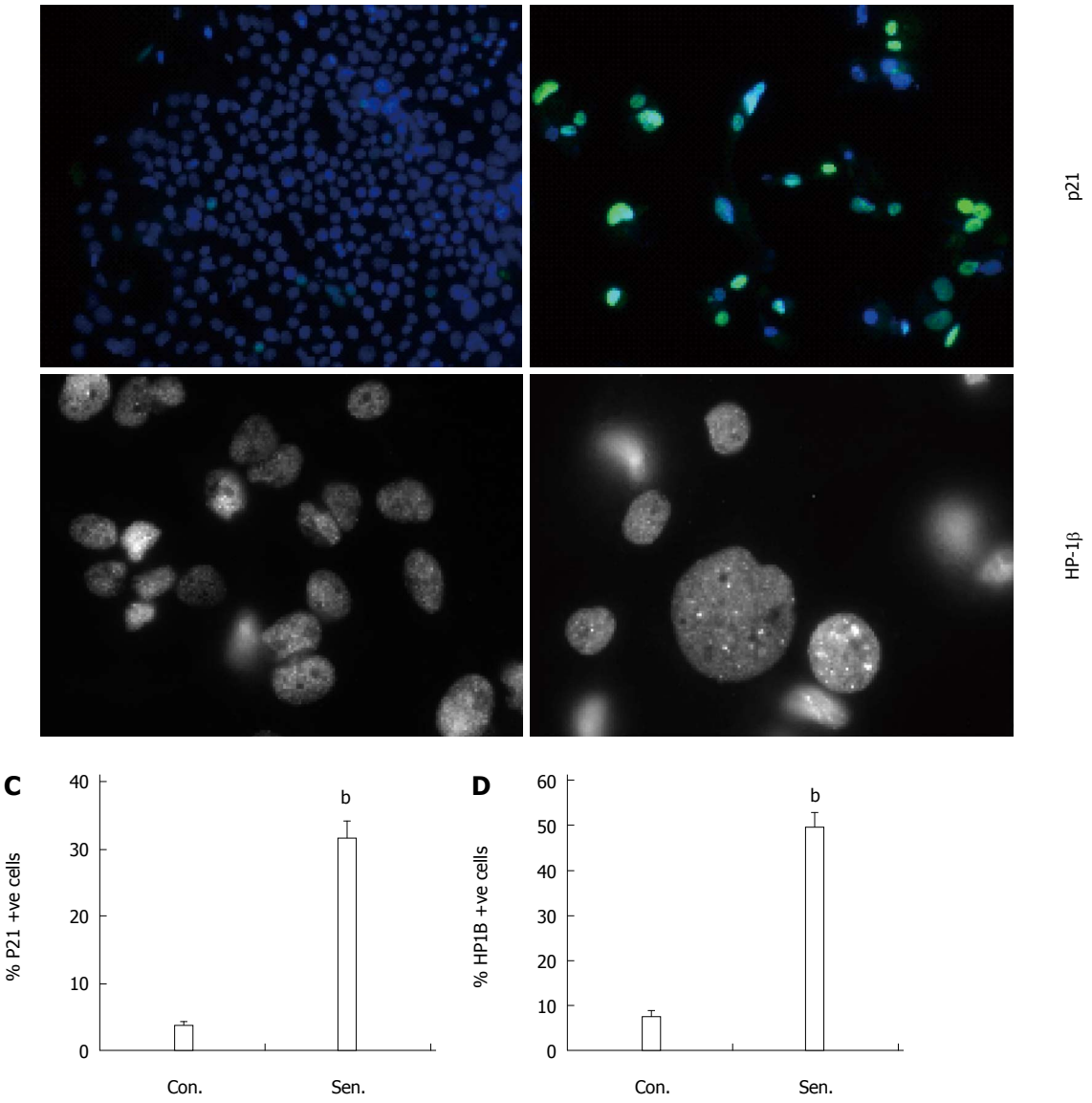

Figure 1 Stress-induced senescence in human hepatocytes. A: HepG2 cells were treated with the indicated doses of hydrogen peroxide (H_2O_2) and survival assessed using a tetrazolium reduction assay; B: Phase contrast, Ki67 immunocytochemistry, β-galactosidase activity, p21 immunocytochemistry, and heterochromatin protein 1 beta (HP1β) immunocytochemistry in control (Con.) and H_2O_2-treated senescent (Sen.) HepG2 7 d after release from H_2O_2 treatment. All panels magnification × 200, except HP-1β magnification × 630; C: Quantification of $p21^+$ nuclei; D: Quantification of cells with $HP1\beta^+$ foci. Data are representative of at least 3 independent experiments. $^bP < 0.01$ *vs* control group.

Transcriptome-based characterisation of the human hepatocyte SASP

Previous studies of cellular senescence have demonstrated that SASP-associated proteins are generally regulated at the level of transcription[15]. In order to gain insight into the hepatocyte SASP we used microarrays to identify changes in gene expression associated with hepatocyte senescence. Senescent and control untreated HepG2 cells were harvested on D10 of the experimental protocol for Illumina expression profiling. Untreated HepG2 cultures harvested on D2 were considered as baseline expression. Both untreated and H_2O_2-treated cells showed significant changes in gene expression after 10 d culture (Figure 3A), however, senescence induced unique mRNA expression changes in HepG2 cells (326 probes, including *CDKN1A* (which encodes p21). Probes that were uniquely regulated in senescent cultures are clustered in Figure 3B, whereas commonly regulated probes are clustered in Figure 3C. As is apparent from Figure 3B, senescence was primarily associated with gene induction. Not surprisingly, *GSTA1* and *GSTA2*, encoding glutathione *S*-transferase enzymes that protect cells against oxidative stress, were among the most highly induced transcripts, although these enzymes have not been associated with senescence in other cell types. Gene ontology enrichment analysis of senescence-induced probes confirmed the increased expression of genes encoding secreted proteins (Table 1). Consistent with other senescent cell models, inflammatory/defence response genes were also highly enriched in the senescent cells, however the enrichment of lipid and drug metabolism pathways has not been previously reported and may be unique to

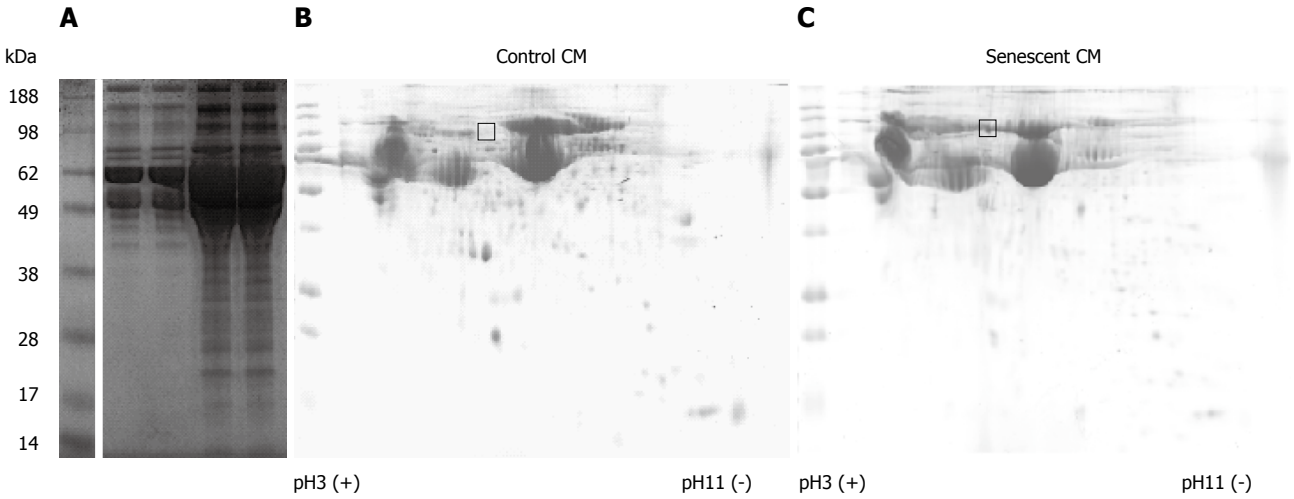

Figure 2 Secretory profile of control and senescent hepatocytes. Proteins in conditioned media (CM) derived from an equivalent number of control (Con.) and senescent (Sen.) hepatocytes were separated by 1-dimensional polyacrylamide gel electrophoresis (PAGE) (A). 150 μg protein from control (B) and senescent (C) hepatocyte conditioned media was separated by 2-dimensional PAGE. Gels were stained with colloidal coomassie. The gel region excised for liquid chromatography mass spectrometry services analysis is indicated. Gel images are representative of 2 independent experiments.

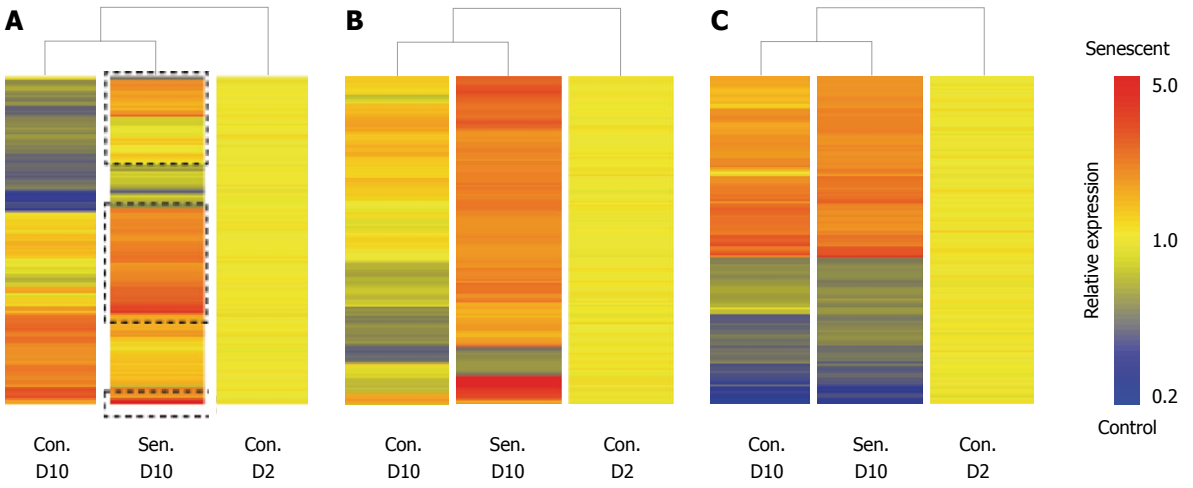

Figure 3 Senescent hepatocyte gene expression signature. Senescent hepatocytes (Sen.) were compared to untreated hepatocytes (Con.) at D (Day) 2 and D10 of the experimental protocol by illumina microarray. A: Hierarchical clustering of probes that were differentially expressed between control and senescent hepatocytes at D10; B: Hierarchical clustering of probes that were uniquely regulated in senescent hepatocytes; C: Hierarchical clustering of probes that were commonly regulated in senescent hepatocytes and untreated hepatocytes between D2 and D10. Probes are clustered by distance correlation with average linkage.

hepatocytes (Table 1). Several gene ontologies were enriched among the 37 genes encoding annotated secreted proteins, which likely contribute to the hepatocyte SASP, including plasma components and proteins involved in the immune response (Table 1). These include the typical SASP component *IL8*, mRNAs from other functional families typically associated with senescence (*e.g.*, *IGFBP7*[20], *SERPINA6*), and acute phase proteins that may be unique to the hepatocyte SASP, including complement 1S, haptoglobin and hemopexin. Commonly regulated genes were broadly associated with cell cycle and metabolism, consistent with a degree of replicative exhaustion and possibly a quiescent state in control cells after prolonged culture (*e.g.*, the proliferation-associated *PCNA* was downregulated in both control and senescent cells). Nevertheless, the cardinal features of senescence were only observed in H_2O_2-treated hepatocytes (Figure 1).

To confirm the results of the microarray, qPCR was performed to assess the expression of selected genes in senescent HepG2 cells. Genes were chosen based on degree of regulation on the microarray and their potential biological relevance. Figure 4 demonstrates the increased expression of *SAA4*, *ECADH*, *IL32* and *IL8* (the latter a frequent SASP feature[15]) in senescent hepatocytes 8 d after release from H_2O_2 treatment. We also quantified *IL6* mRNA expression by qPCR, since it is a key senescence-associated gene[11], but was not detectable by microarray. IL6 was below the limit of detection in 2/4 control HepG2 cultures, however it was induced in se-

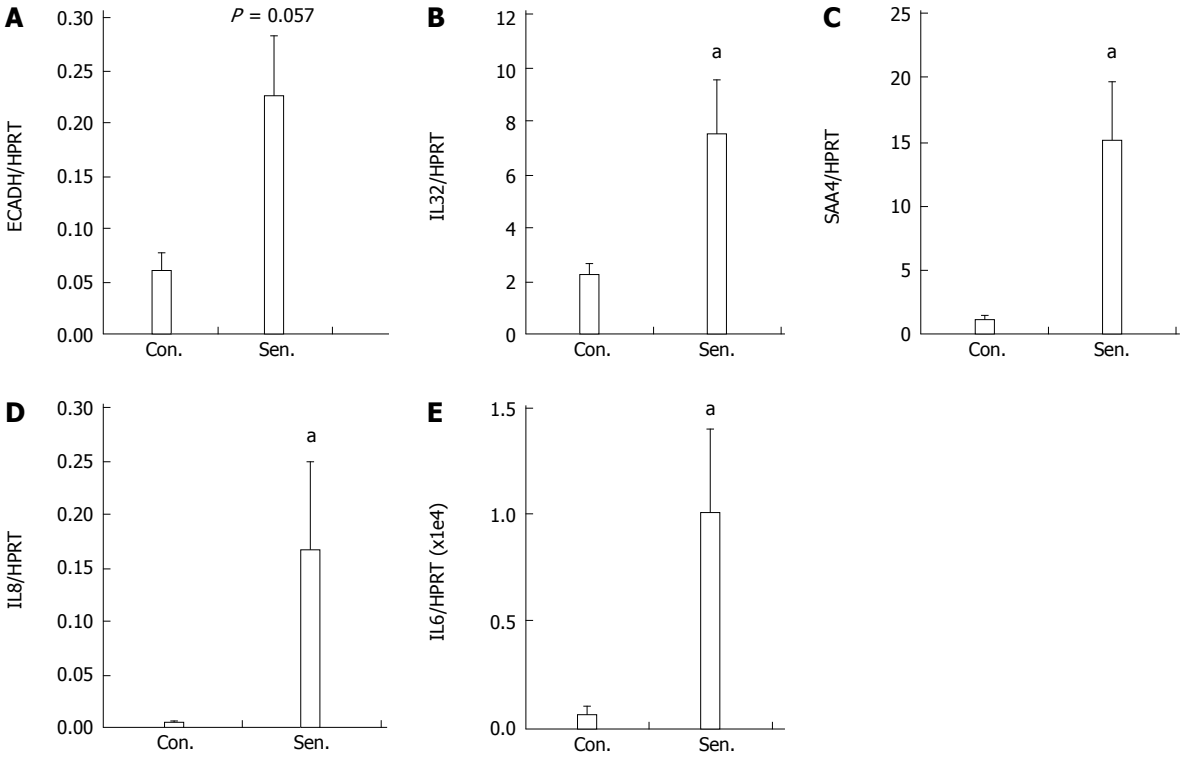

Figure 4 Quantitative polymerase chain reaction validation of microarray. Relative mRNA expression of A: *ECADH*; B: *IL32*; C: *IL8*; D: *SAA4* and E: *IL6* in control (Con.) and senescent (Sen.) hepatocytes was quantified by real-time polymerase chain reaction PCR. Data represent mean + SEM, $n = 4$ independent experiments. $^{a}P < 0.05$ *vs* control group.

Table 1 Gene ontology analysis of senescent hepatocyte gene expression signature

Gene list	Ontology	% list	*P* value	Fold enrichment
A: senescence induced	Steroid metabolism	8.5	5.50E-12	7.4
	Endoplasmic reticulum	15.7	7.10E-9	2.8
	Oxidation/reduction	12.1	8.00E-9	3.5
	Drug metabolism	4.8	7.80E-8	8.6
	Lysosome	6.0	2.20E-6	4.9
	Retinol metabolism	4.0	2.20E-6	8.3
	Signal peptide	27.8	1.40E-5	1.7
	Iron ion binding	6.5	3.20E-5	3.6
	Defence response	8.5	3.00E-4	2.5
	Lipid biosynthesis	5.6	5.40E-4	3.1
	Lipid catabolism	4.0	7.20E-4	4.1
	Secreted	14.9	1.50E-3	1.7
	Regulation of apoptosis	9.3	1.90E-3	2.0
	Inflammatory response	4.4	1.50E-2	2.4
B: secreted	Disulfide bond	62.2	3.90E-10	4.1
	Plasma	16.2	9.10E-7	32.6
	Glycoprotein	56.8	2.10E-5	2.5
	Defence response	21.6	2.10E-4	6.0
	Immune response	16.2	1.10E-2	4.2
	Lipid binding	13.5	1.40E-2	5.0
	Cell adhesion	13.5	5.80E-2	3.3
	Regulation of proliferation	13.5	8.30E-2	2.9

Gene ontology enrichment in (A) senescence-induced genes and (B) senescence-induced genes falling in the "Secreted" ontology was analysed using DAVID.

nescent hepatocytes (Figure 4E).

Chemokine profile of senescent hepatocytes

In order to focus on chemokines secreted by senescent hepatocytes, since these low abundance proteins are likely to be masked by abundant secretory products (Figure 2), we used a protein array to investigate the expression of 31 chemokines in control and senescent CM (equivalent

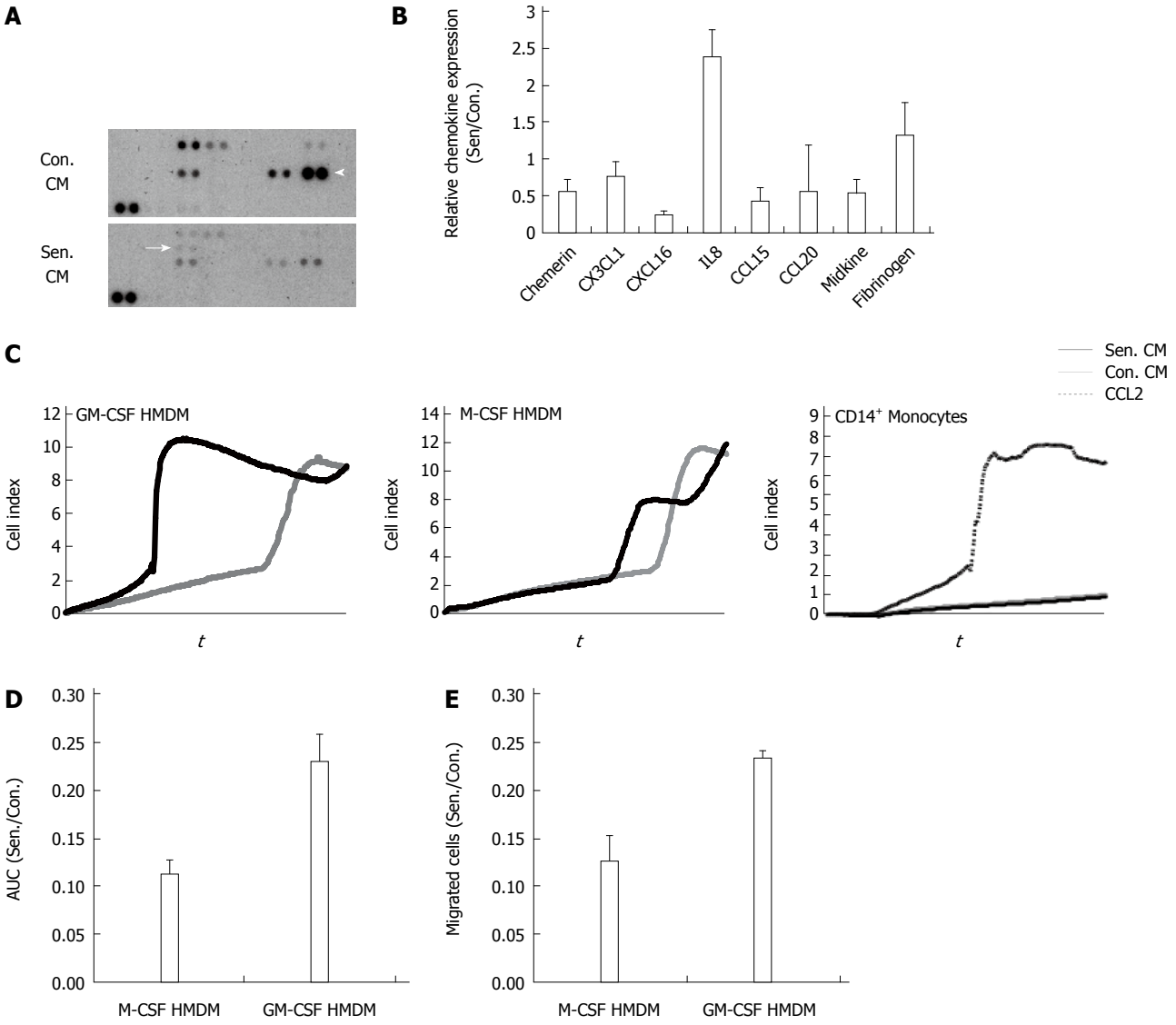

Figure 5 Chemokine profile and chemotactic effects of senescent hepatocyte conditioned medium. A: Representative human chemokine array. Arrow: IL8. Arrow head: CCL20; B: Relative protein expression in control and senescent conditioned medium (CM). Data show the average + range of 2 independent experiments; C: Representative xCELLigence trace, macrophage and monocyte migration in response to control and senescent CM and recombinant CC chemokine ligand 2 (CCL2); D: Relative migration of macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF)-derived human monocyte derived macrophages (HMDM) in senescent (Sen.) compared to control (Con.) CM in D: xCELLigence assay quantified by the area under the curve (AUC); E: Transwell assay, quantified by counting migrated nuclei. Data represent mean + SE.

volume). Seven chemokines were detectable in hepatocyte CM (Figure 5A, B); prominent among them were CCL20 (also known as liver activation regulated chemokine) and CXCL16. As shown at the mRNA level (Figure 4) IL-8, was present at higher levels in senescent CM (Figure 5A, B). Consistent with the microarray there was also a modest increase in fibrinogen (included on the chemokine array as a sample control due to its broad expression), which showed significant upregulation of *FGB* in senescent cells. Since senescent cell cultures contained on average 5-fold fewer cells, considering chemokine levels on a per cell basis would further amplify the difference between control and senescent CM. The relatively reduced expression of CXCL16 on the chemokine array is also consistent with the microarray, in which *CXCL16* mRNA was induced during culture in control but not senescent cells.

Senescent hepatocyte conditioned medium induces macrophage migration

Senescent hepatocytes have been implicated in fibrogenesis and chronic liver disease progression, both directly and *via* recruitment of inflammatory cells, especially macrophages[21]. We investigated monocyte and monocyte-derived macrophage migration towards control and senescent HepG2 CM using the xCELLigence system to monitor cell migration in real time (Figure 5C). Primary peripheral blood monocytes did not respond to either CM, but did migrate to the chemokine CCL2, which was used as a positive control. By contrast, HMDM differentiated in the presence of M-CSF or GM-CSF migrated in response to an equivalent volume of control and se-

nescent CM (Figure 5C). Whilst senescent CM did not alter M-CSF-derived macrophage migration, there was a significant increase in GM-CSF-derived macrophage migration towards senescent CM compared to control CM (Figure 5C and 5D, $P = 0.022$). The contribution of morphological change to the xCELLigence signal cannot be discounted, as this system simply measures electrical impedance. We therefore used conventional transwell assays to confirm differential migration of GM-CSF- but not M-CSF-derived macrophages towards senescent CM (Figure 5E). As discussed above, the increased macrophage migration to senescent cell secretory products is even greater when considered on a per cell basis.

DISCUSSION

Senescent hepatocytes are prominent in chronic liver diseases, but their "secretory phenotype" and contribution to the tissue microenvironment has not been characterized. In this study, premature senescence was induced in HepG2 cells by oxidative stress, providing a novel model to examine hepatocyte senescence. Treatment with a sublethal dose of H_2O_2 provides a relevant *in vitro* model of hepatocyte senescence as oxidative stress with the generation of reactive oxygen species, including H_2O_2, is a key feature of many chronic liver diseases[22], Reactive oxygen species-induced senescence is mediated by both the characterised pathways to senescence - p53/p21 and p16/Rb[10]. In the current model, hepatocytes treated with 300 μmol/L H_2O_2 for 48 h developed a senescent phenotype, which persisted for at least 7 d after release from treatment. The treated cells acquired several markers of senescence, namely reduced Ki67 expression, the appearance of SAHF and p21 and SA-β-Gal positivity[10]. We used our model to profile the hepatocyte SASP, demonstrating that it shares features with other senescent cells, and that it promotes inflammatory macrophage migration.

Although the focus of this study is hepatocyte senescence and its potential contribution to chronic liver disease, senescence in the liver is clearly a complex issue. Other liver cell types, notably activated hepatic stellate cells[12] and hepatic progenitor cells[19], have been reported to senesce, and the outcomes of senescence may differ depending on cell type and context. Senescence and immune-mediated clearance of activated HSC, for example, was shown to limit fibrosis in mice[12], whilst HSC senescence promoted obesity-associated hepatocellular cancer[23]. Hepatocyte senescence may likewise have protective and pathogenic functions; as defective immune clearance of pre-malignant senescent hepatocytes was associated with early development of liver cancer[21,24], whereas triggering senescence by reactivation of endogenous *p53* mediated clearance of liver tumours[25]. Interestingly, HCV core protein specifically targets the *p16* senescence response, suggesting hepatocyte senescence is employed as a strategy to limit viral replication[26].

Increasing evidence suggests senescent cells contribute to the pathogenesis of chronic disease by adopting a characteristic secretory phenotype, known as the SASP, which alters the tissue microenvironment[11,15]. This is consistent with the strong 'secreted protein' signature we observed upon expression profiling senescent hepatocytes, and their increased protein production compared to control cells. Senescent hepatocytes upregulated expression of inflammatory cytokines/chemokines, matrix-remodeling proteases and growth factors; and also display increased expression of genes accordant with their liver-specific cellular function such as lipoproteins and cholesterol metabolism. Many of these changes in gene expression are seen in other senescent cell types (fibroblasts and epithelial cells), consistent with a conserved core phenotype that may be controlled at a transcriptional level in response to genotoxic stress[15].

IL-8 is one of the most prominent components of the SASP - regardless of cell type and senescence trigger. Several studies in human chronic liver disease suggest a link between IL-8 and liver injury. In chronic hepatitis C and B infection, serum IL-8 levels increase progressively with severity of liver disease[27,28] and development of hepatocellular cancer[27,29]. Although hepatocyte senescence was not examined in these earlier studies, we and others have previously shown that many hepatocytes in liver sections from patients with NAFLD[30,31], HCV[5], and hepatocellular cancer[7] are senescent, as assessed by nuclear p21 or SA-β-Gal expression. The biologic effects of IL-8 in the microenvironment of senescent hepatocytes have not been determined. IL-8 production by senescent cells has been shown to reinforce growth arrest[32], stimulate angiogenesis and promote tumorogenesis[33]. IL-8, together with IL-6, has also been implicated in eliciting EMT in senescent cells, however we did not detect a signature consistent with EMT in senescent HepG2 cells, in fact *ECADH* was upregulated. IL-8 is also a potent leukocyte chemoattractant, which may play a role in recruiting macrophages or neutrophils to senescent cells. Elevated intrahepatic IL-8 and CXCR1 levels were associated with hepatic macrophage accumulation in hepatocellular, but not cholestatic, chronic liver diseases, in parallel with increased CXCR1 expression on circulating monocytes[28]. We demonstrated that senescent hepatocyte CM enhanced migration of HMDM differentiated in the presence of the inflammatory cytokine GM-CSF, but not the constitutive macrophage growth factor, M-CSF, or monocytes. This finding is consistent with our recent demonstration of elevated *IL8RA/CXCR2* mRNA in GM-CSF-derived HMDM, as compared to those derived with M-CSF[34], and suggests senescent hepatocytes modulate the inflammatory milieu by selectively recruiting specific immune cells, *via* IL-8 and/or other SASP components. Indeed, defective immune surveillance of senescent cells exacerbated liver injury and fibrosis, and tumour development in mouse models of liver disease[12,21]. Increased numbers of senescent hepatocytes were also observed in explant livers from immunosuppressed HCV patients[21].

In summary, we have developed an *in vitro* model

of human hepatocyte senescence and, for the first time, characterised the hepatocyte SASP using a transcriptomic approach. Senescent hepatocytes upregulate characteristic SASP factors such as IL-8, and selectively promote recruitment of inflammatory (GM-CSF-derived) macrophages. SASP-mediated recruitment of inflammatory cells may be a key mechanism by which senescence contributes to injury resolution or pathogenesis in chronic liver disease. Future studies on novel SASP factors that we have identified are likely to provide mechanistic insights into such processes.

COMMENTS

Background

Cellular senescence, a form of permanent replicative arrest, is a phenomenon associated with aging, inflammation and cancer. Although widely studied in cancer biology, the role of cellular senescence in the pathogenesis of inflammatory diseases, such as chronic liver disease, is not known. Hepatocyte senescence is frequently observed in all chronic liver diseases of hepatocellular origin, even at early stages of disease progression. It is important to understand the impact of hepatocyte senescence on liver disease, since it may have both protective and pathological roles.

Research frontiers

The global burden of liver disease is steadily increasing, particularly liver cancer, which develops in the setting of chronic disease. Hepatocyte senescence is associated with progression of chronic liver disease and the development of liver cancer, but how senescence contributes to disease outcomes is not well understood.

Innovations and breakthroughs

Authors developed a novel model of stress-induced hepatocyte senescence and used it to demonstrate that senescent human hepatocytes adopt a hypersecretory phenotype, which is likely to condition their microenvironment and contribute to disease pathogenesis. They used microarray and proteomic analysis to characterise senescent hepatocytes and identify candidate mediators; and confirmed the functional relevance of senescence-associated secretory phenotype by demonstrating that conditioned media from senescent hepatocytes elicits inflammatory macrophage migration.

Applications

The results of this study suggest senescent hepatocytes recruit inflammatory macrophages, and identify candidate mediators of this process, which represent targets for validation *in vivo*. Their novel model will facilitate investigations into the mechanistic basis of inflammatory cell recruitment, and the impact of senescent hepatocyte secretory products on diverse cell types implicated in liver disease progression.

Peer review

The authors developed a great model of human hepatocyte senescence and they characterised the hepatocyte senescence associated secretory phenotype (SASP) using a transcriptomic approach. Senescent hepatocytes upregulate characteristic SASP factors such as IL-8, and selectively promote recruitment of macrophages. SASP-mediated recruitment of inflammatory cells may be a key mechanism by which senescence contributes to injury resolution or pathogenesis in chronic liver disease.

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ORIGINAL ARTICLE

Claudin 1 mediates tumor necrosis factor alpha-induced cell migration in human gastric cancer cells

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Author contributions: Shiozaki A and Shimizu H contributed equally to this work; Shiozaki A and Shimizu H designed the study, wrote the manuscript and performed the majority of the experiments; Ichikawa D, Konishi H, Komatsu S, Kubota T, Fujiwara H and Okamoto K analyzed the microarray data; Iitaka D, Nakashima S and Nako Y analyzed cell migration and invasion; Liu M and Otsuji E were involved in editing the manuscript.

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Abstract

AIM: To investigate the role of claudin 1 in the regulation of genes involved in cell migration and tumor necrosis factor alpha (TNF-α)-induced gene expression in human gastric adenocarcinoma cells.

METHODS: Knockdown experiments were conducted with claudin 1 small interfering RNA (siRNA), and the effects on the cell cycle, apoptosis, migration and invasion were analyzed in human gastric adenocarcinoma MKN28 cells. The gene expression profiles of cells were analyzed by microarray and bioinformatics.

RESULTS: The knockdown of claudin 1 significantly inhibited cell proliferation, migration and invasion, and increased apoptosis. Microarray analysis identified 245 genes whose expression levels were altered by the knockdown of claudin 1. Pathway analysis showed that the top-ranked molecular and cellular function was the cellular movement related pathway, which involved MMP7, TNF-SF10, TGFBR1, and CCL2. Furthermore, TNF- and nuclear frctor-κB were the top-ranked upstream regulators related to claudin 1. TNF-α treatment increased claudin 1 expression and cell migration in MKN28 cells. Microarray analysis indicated that the depletion of claudin 1 inhibited 80% of the TNF-α-induced mRNA expression changes. Further, TNF-α did not enhance cell migration in the claudin 1 siRNA transfected cells.

CONCLUSION: These results suggest that claudin 1 is an important messenger that regulates TNF-α-induced gene expression and migration in gastric cancer cells. A deeper understanding of these cellular processes may be helpful in establishing new therapeutic strategies for gastric cancer.

Key words: Tumor necrosis factor alpha; Claudin 1; Cell migration; Microarray; Gene expression change

Core tip: The objectives of the present research were to investigate the role of claudin 1 in the regulation of genes involved in cell migration and tumor necrosis factor alpha (TNF-α)-induced gene expression in human gastric adenocarcinoma cells. Claudin 1 small interfering RNA transfection significantly inhibited cell migration and invasion in gastric cancer cells. Micro-

array analyses showed that down-regulation of claudin 1changed the expression levels of many genes related to cellular movement and TNF-α signal. We showed that TNF-α treatment induces the expression of claudin 1 in gastric carcinoma cells, and the latter controls gene expression and cell migration as the signal mediator.

Shiozaki A, Shimizu H, Ichikawa D, Konishi H, Komatsu S, Kubota T, Fujiwara H, Okamoto K, Iitaka D, Nakashima S, Nako Y, Liu M, Otsuji E. Claudin 1 mediates tumor necrosis factor alpha-induced cell migration in human gastric cancer cells. *World J Gastroenterol* 2014; 20(47): 17863-17876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17863.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17863

INTRODUCTION

Claudin proteins play an essential role in the function of tight junction (TJ) and the maintenance of the polarity of epithelial cells, and 24 subtypes of the claudin have been identified[1-3]. On the other hand, it has been shown that claudin 1 may influence the biological behavior of tumor progression[4,5]. The expression of claudin 1 increases during tumor development of various types of neoplasm including gastrointestinal cancer[6-10]. Further, expression of claudin 1 has prognostic impact in colon cancer and is related to apoptosis in breast cancer cells[11-13]. In gastric cancer, it has been reported that regulation of claudin 1 is related to transformation in invasive front and metastatic lesion[6,13].

Tumor necrosis factor alpha (TNF-α) is involved in epithelial-mesenchymal transition (EMT)[14] and related to the malignant behavior of epithelial tumors by regulating cell migration and invasion. The progression of EMT usually decreases the expression of TJ and adherens junction proteins, such as claudins, occludins and E-Cadherin[15-20]. However, we recently found that TNF-α treatment increased the expression of claudin 1 in human lung carcinoma A549 cells[21]. Further studies showed that claudin 1 plays an important role in TNF-α-induced gene expression and cellular movement in A549 cells[21].

The objective of the present research was to investigate the role of claudin 1 in the regulation of genes involved in migration and TNF-α-induced gene expression in human gastric adenocarcinoma cells. Our results showed claudin 1 small interfering RNA (siRNA) transfection significantly inhibited cell migration and invasion in gastric cancer cells. Furthermore, microarray analyses showed that down-regulation of claudin 1changed the expression levels of many genes related to cellular movement and TNF-α signal. Our results indicate that claudin 1 plays an important role in TNF-α-induced gene expression and cellular movement in gastric carcinoma cells.

MATERIALS AND METHODS

Cell lines, antibodies, and other reagents

The human gastric adenocarcinoma cell lines MKN28, NUGC4, MKN45 and Kato-Ⅲ were grown in plastic culture flasks (Corning Incorporated, NY, United States) and maintained in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 μg/mL of streptomycin, as described previously[22]. The flasks were kept in a humidified incubator at 37 ℃ under 5.0% CO_2[22].

The following antibodies were used in the study; claudin 1 antibody (Zymed Laboratories, San Francisco, CA), a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling Technology, Beverly, MA), and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Human TNF-α was from R&D Systemic Inc. (Minneapolis, MN).

Protein studies

Cells were harvested in M-PER lysis buffer supplemented with protease inhibitors (Pierce, Rockford, IL). The protein concentration was measured with a modified Bradford assay (Bio-Rad, Hercules, CA)[23]. Cell lysates containing equal amounts of total protein were separated by SDS-PAGE and then were transferred onto PVDF membranes (GE Healthcare, Piscataway, NJ)[23]. The membranes were probed with the indicated antibodies, and proteins were detected by the ECL Plus Western Blotting Detection System (GE Healthcare)[23,24].

siRNA transfection

Cells were transfected with 10 nmol/L claudin 1 siRNA (Santa Cruz) using the Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA). The medium containing the siRNA was replaced with fresh medium after 24 h[23,24]. The provided control siRNA (Santa Cruz) was used as a negative control. The siRNA transfected cells were harvested 48 h after transfection for protein studies[23,24]. Further, we used a second independent claudin 1 siRNA, Stealth RNAi siRNA targeting claudin 1 mRNA (Invitrogen) to exclude off target effects.

Real-time quantitative RT-PCR

Total RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA)[24]. Messenger RNA (mRNA) expression was measured by a quantitative real-time PCR (7300 Real-Time PCR System; Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays (Applied Biosystems). The expression level of claudin-1 gene (Hs00221623_m1; Applied Biosystems) was measured, and normalized against the housekeeping gene beta-actin (ACTB, Hs01060665_g1; Applied Biosystems). Each assay was performed in triplicate.

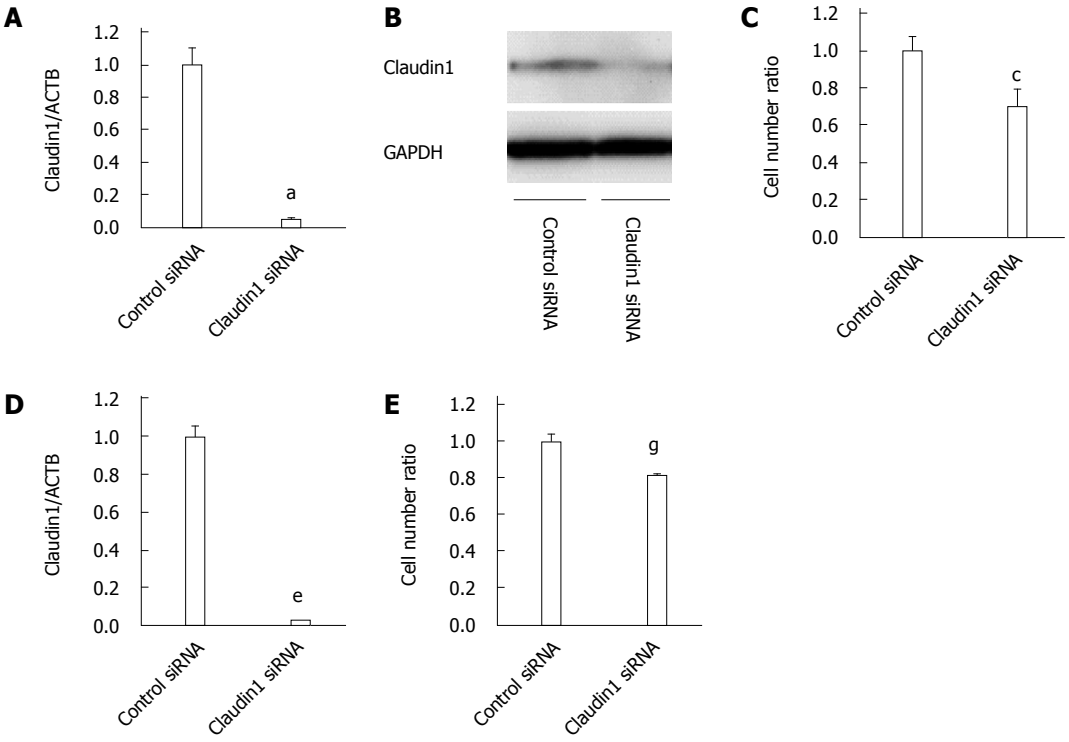


Figure 1 Claudin 1 controls cell proliferation in MKN28 cells. A: Claudin 1 siRNA effectively reduced the mRNA levels of claudin 1 in MKN28 cells. Mean ± SE. $n = 3$. [a]$P < 0.05$ *vs* control siRNA; B: Western blotting revealed that claudin 1 siRNA reduced the protein levels of claudin 1 in MKN28 cells; C: Downregulation of claudin 1 inhibited the proliferation of MKN28 cells. The number of cells was counted 48 h after siRNA transfection. Mean ± SE. $n = 3$. [c]$P < 0.05$ *vs* control siRNA; D: A second independent claudin 1 siRNA also reduced the mRNA levels of claudin 1 in the MKN28 cells. Mean ± SE. $n = 3$. [e]$P < 0.05$ *vs* control siRNA; E: A second independent claudin 1 siRNA also inhibited the proliferation of MKN28 cells. The number of cells was counted 48 h after siRNA transfection. Mean ± SE. $n = 3$. [g]$P < 0.05$ *vs* control siRNA.

Cell proliferation

Cells were seeded onto 6 well plastic plates at a density of 1.0×10^5 cells per well and incubated at 37 ℃ with 5% CO_2. At 24 h after the cell seeding, siRNA transfection was performed. At 48 h after siRNA transfection, the cells were detached from the plates using a trypsin-EDTA and were counted using Countess® Automated Cell Counter (Invitrogen). Each sample was independently counted for three times, and each assay was performed in triplicate.

Analysis of apoptotic cells

As control, non-transfected cells were treated with staurosporine for 24 h. At 48 h after transfection, the siRNA transfected cells were harvested and stained with fluorescein isothiocyanate (FITC) conjugated annexin V and phosphatidylinositol using the annexin V Kit (Beckman Coulter, Brea, CA)[23]. A Becton Dickinson Accuri™ C6 Flow Cytometer was used to analyze the proportion of apoptotic cells.

Analysis of cell migration and invasion

The migration assay was conducted using a cell culture insert with 8 μm pore size (BD Biosciences, Bedford, MA). Biocoat Matrigel (BD Biosciences) was used to evaluate cell invasion potential. Briefly, at 24 h after siRNA transfection, cells (2.0×10^4 cells per well) were seeded in the upper chamber in serum free medium[25]. The lower chamber contained medium with 10% FBS. The chambers were incubated for 48 h for migration assay and 60 h for invasion assay at 37 ℃ in 5% CO_2, and then, non-migrated or non-invaded cells were removed from the upper side of the membrane by scrubbing with cotton swabs[25]. Migrated or invaded cells were fixed on the membrane and stained with Diff-Quick staining reagents (Sysmex, Kobe, Japan)[25]. The migrated or invaded cells on the lower side of the membrane were counted in four independent fields of view at magnification × 100 of each insert[25]. Each assay was performed in triplicate.

Microarray sample preparation and hybridization

MKN28 cells were transfected with control siRNA and claudin 1 siRNA. At 48 h after siRNA transfection, total RNA was extracted using an RNeasy kit (Qiagen). Quality of RNA was monitored using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Cyanine-3 (Cy3)-labeled cRNA was prepared from 0.1 μg total RNA using the Low Input Quick Amp Labeling Kit (Agilent). Samples were purified using RNAeasy columns (Qiagen). A total of 0.60 μg of Cy3-labelled cRNA was fragmented and hybridized to an Agilent SurePrint G3 Human Gene Expression 8 × 60K Microarray for 17 h. After washing, slides were scanned on the Agilent DNA Microarray Scanner (G2565CA) using the one color scan setting for 8 K × 60 K array slides.

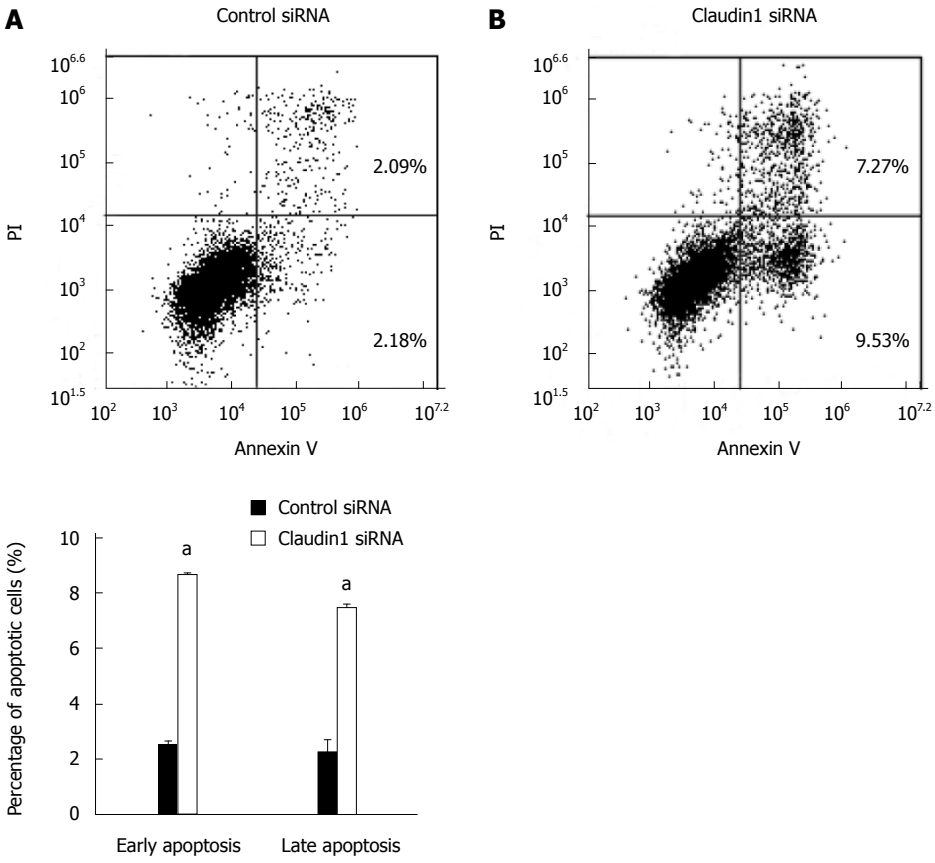


Figure 2 Claudin 1 controls apoptosis in MKN28 cells. Down-regulation of claudin 1 induced both early (annexin V positive/PI negative) and late apoptosis (annexin V/PI double positive) in MKN28 cells 48 h after siRNA transfection. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA.

Processing of microarray data

The scanned images were analyzed with Feature Extraction Software 10.10 (Agilent) using default parameters to obtain background subtracted and spatially detrended Processed Signal intensities[26]. The fold change of each molecule was calculated by using raw signal data of two samples, and a fold change cutoff of 5 was set to identify molecules whose expression was significantly differentially regulated. The networks and functional analyses were generated using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc., Redwood City, CA).

Immunofluorescent Staining and Confocal Microscopy

MKN28 cells were cultured on glass coverslips and stained as previously described[21]. After different stimulation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% BSA, stained with designated antibodies and rhodamine phalloidin. Slides were mounted with VECTASHIELD mounting medium and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, United States), and distribution of Claudin 1 was examined by confocal fluorescence microscopy (LSM510; Carl Zeiss Co. Ltd, Germany).

Statistical analysis

Student's *t*-test was used to evaluate continuous variables. Differences were considered significant when the relevant *P* value was < 0.05. These analyses were performed using JMP version 10 (SAS Institute Inc., Cary, NC).

RESULTS

Claudin 1 controlled cell proliferation, apoptosis, migration and invasion in MKN28 cells

We conducted knockdown experiments with claudin 1 siRNA in MKN28 cells, and analyzed the effects of claudin 1 knockdown on cell proliferation, apoptosis, migration and invasion. Claudin 1 siRNA effectively reduced claudin 1 mRNA levels (Figure 1A) and claudin 1 protein levels (Figure 1B). The cell counts of claudin 1 siRNA transfected cells were significantly lower than those of control siRNA transfected cells 48 h after siRNA transfection (Figure 1C). Similar results were obtained by using another independent claudin 1 siRNA (Figure 1D, E). Down-regulation of claudin 1 increased both early (annexin V; positive and PI; negative) and late apoptosis (annexin V; positive and PI; positive) 48 h after siRNA transfection (Figure 2). Furthermore, down-regulation of claudin 1 significantly inhibited cell migration and invasion (Figure 3). These results suggest that claudin 1 plays a crucial role in regulating cell proliferation, apoptosis, migration and invasion in MKN28 cells.

Gene expression profile of claudin 1 siRNA transfected cells

We analyzed the gene expression profiles of claudin 1

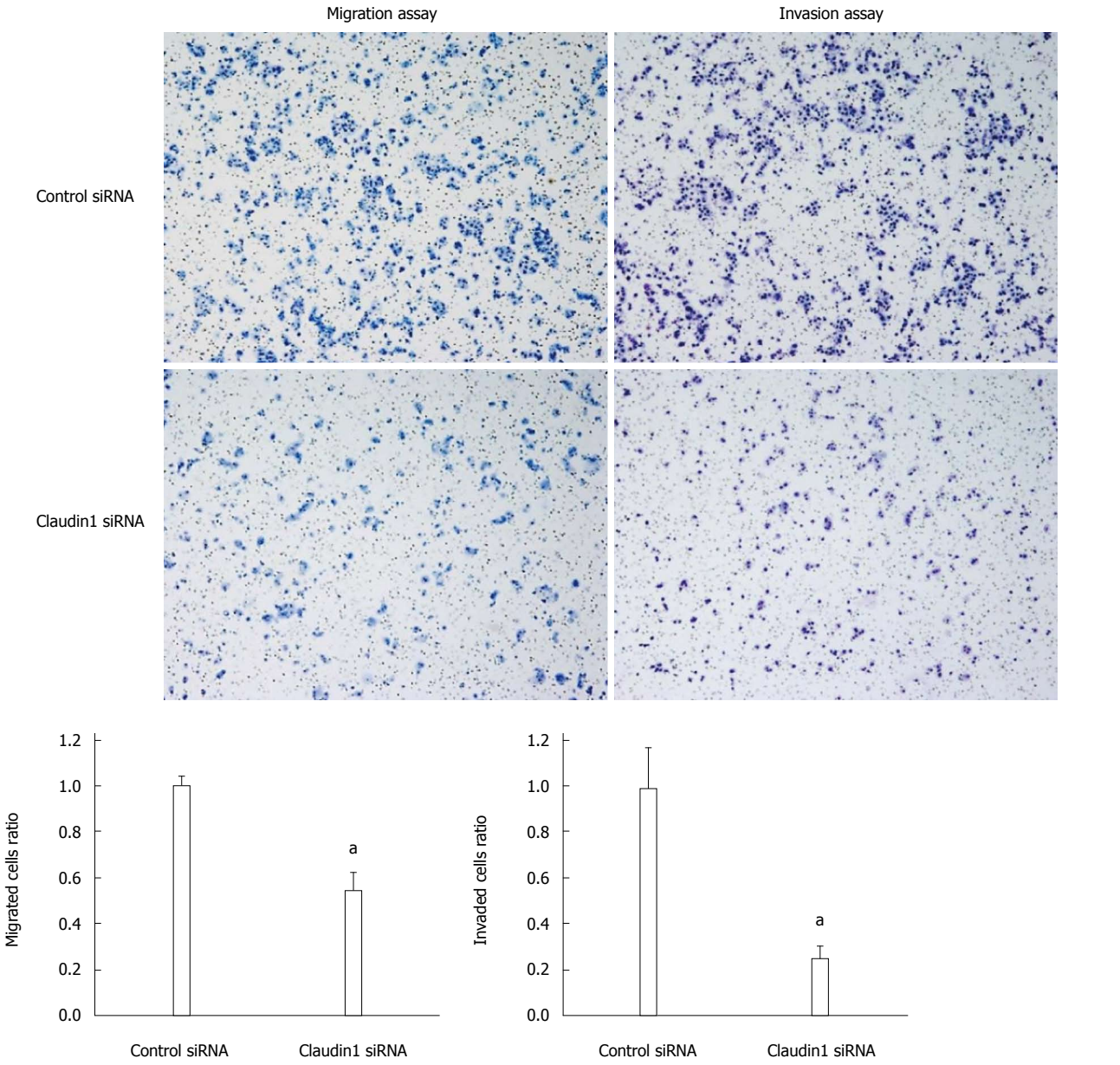


Figure 3 Claudin 1 controlls cell migration and invasion in MKN28 cells. Down-regulation of claudin 1 significantly inhibited cell migration and invasion in MKN28 cells. Cell migration and invasion were determined by Boyden chamber assay. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA.

siRNA transfected MKN28 cells with microarray and bioinformatics. Microarray analysis showed that the expression levels of 245 genes displayed fold changes of > 5.0 in MKN28 cells subjected to claudin 1 knockdown. Of these genes, 76 were upregulated, and 169 were downregulated in claudin 1 siRNA transfected cells. The 20 genes whose expression levels were the most strongly up- or down-regulated in claudin 1 siRNA transfected cells are shown in Table 1. Claudin 1 expression was down-regulated in claudin 1 siRNA transfected cells (fold change: -26.87; Table 1). Ingenuity Pathway Analysis showed that "Cellular movement" was the top-ranked molecular and cellular functions (Table 2(. Furthermore, TNF- and nuclear factor (NF)-κB were the top-ranked upstream regulators related to claudin 1 (Table 2). We then examined the signal transduction networks induced by the knockdown of claudin 1 expression (Table 2). One of the top-ranked signal networks was related to the cellular movement (Table 2, Figure 4A). These results indicate that the expression level of claudin 1 influences genes related to cellular movement, and that TNF-α signal may be its important upstream regulator.

Verification of gene expression by real-time quantitative RT-PCR

Four genes (*MMP7*, *TNF-SF10*, *TGFBR1*, and *CCL2*) were examined further using quantitative RT-PCR. All these genes were chosen from Figure 4A. The expression levels of *MMP7*, *TNF-SF10*, and *TGFBR1* mRNA were significantly lower in claudin 1 siRNA transfected cells than in control siRNA transfected ones (Figure 4B). The expression levels of *CCL2* tended to be increased

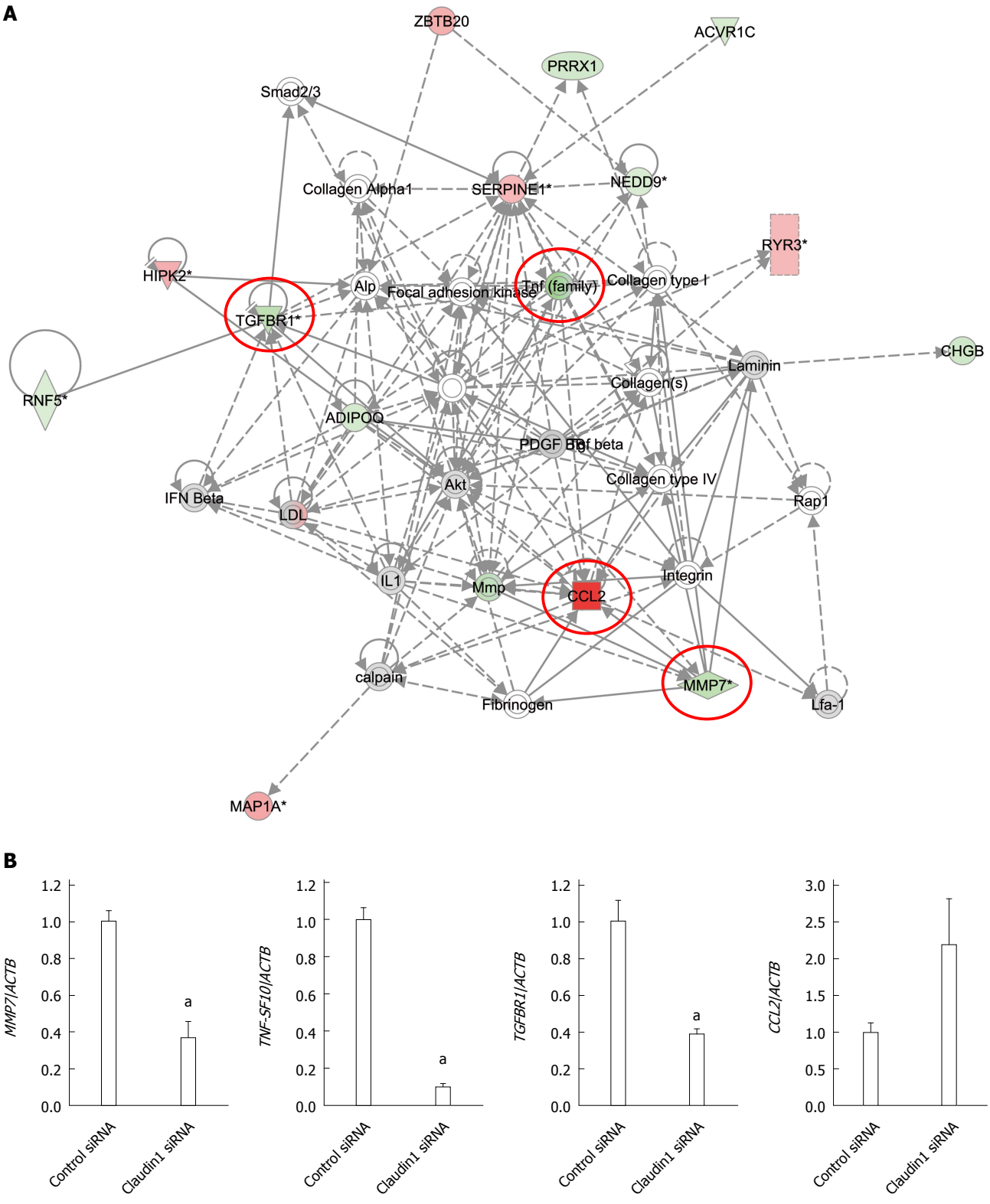


Figure 4 Analysis of gene expression change in claudin 1 siRNA transfected MKN28 cells. A: One of the top-ranked signaling networks related to claudin 1 down-regulation according to ingenuity pathway analysis. Red and green indicate genes whose expression levels were higher or lower, respectively, than reference RNA levels. Genes analyzed for verification were highlighted by red circles; B: Verification of gene expression by real-time quantitative RT-PCR. The expression levels of for selected genes *MMP7*, *TNF-SF10*, *TGFBR1*, and *CCL2* in claudin 1 siRNA transfected MKN28 cells were compared with those in control siRNA transfected cells. Gene expression levels were normalized to the level of *ACTB*. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA.

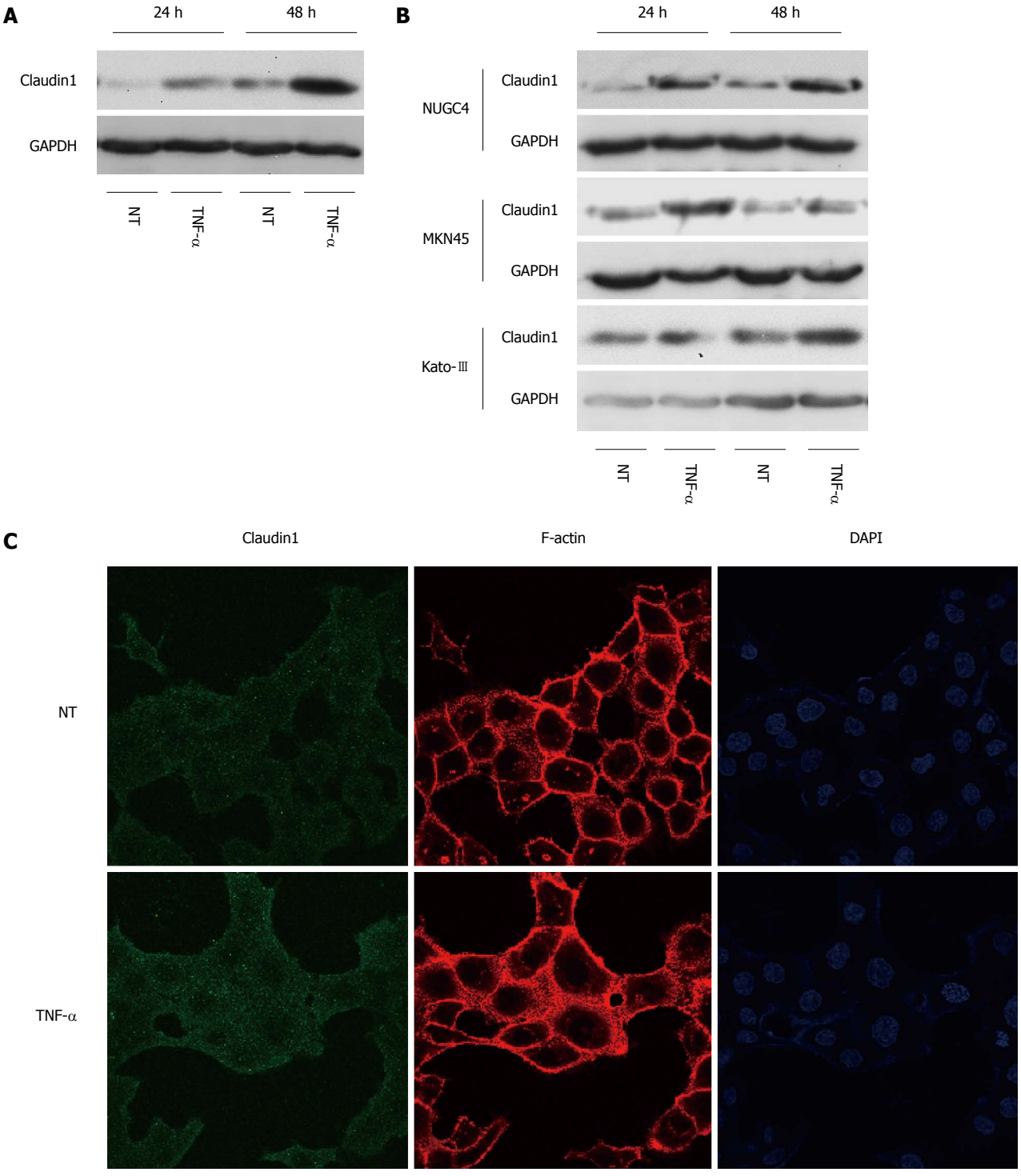
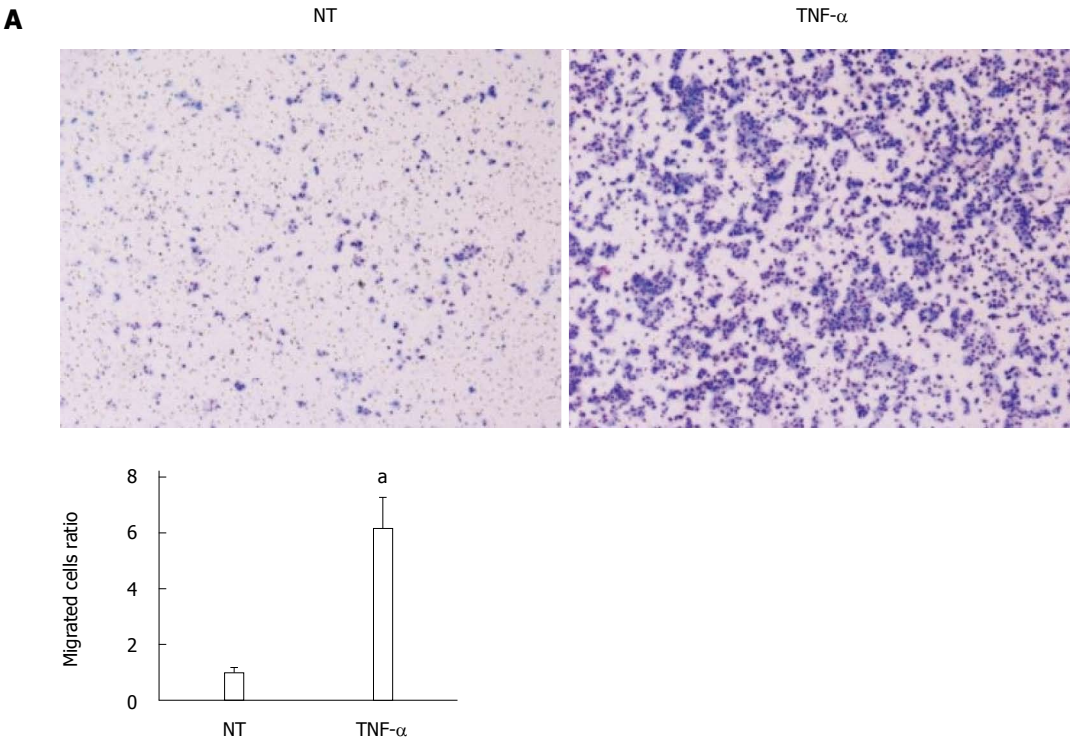


Figure 5 Tumor necrosis factor α induces claudin 1 expression in gastric cancer cells. A: Western blotting revealed that the basal expression level of claudin 1 was increased in a time-dependent manner at 24 and 48 h, which was further increased by tumor necrosis factor α (TNF-α) (20 ng/mL) stimulation in MKN28 cells; B: Western blotting revealed that the expression level of claudin 1 was increased by TNF-α (20 ng/mL) stimulation in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ; C: At 24 h after treating the cells with TNF-α, expression of claudin 1 protein was increased mainly in cytoplasm. MKN28 cells were immunostained with an anti-claudin 1 antibody and counterstained F-actin and nuclei with rhodamine phalloidin and DAPI, respectively.

in claudin 1 siRNA transfected cells although the difference was not statistically significant (Figure 4B). These changes were in agreement with the microarray results.

Claudin 1 plays important roles in TNF-α-induced cell migration in gastric cancer cells

We previously showed that claudin 1 mediates TNF-α-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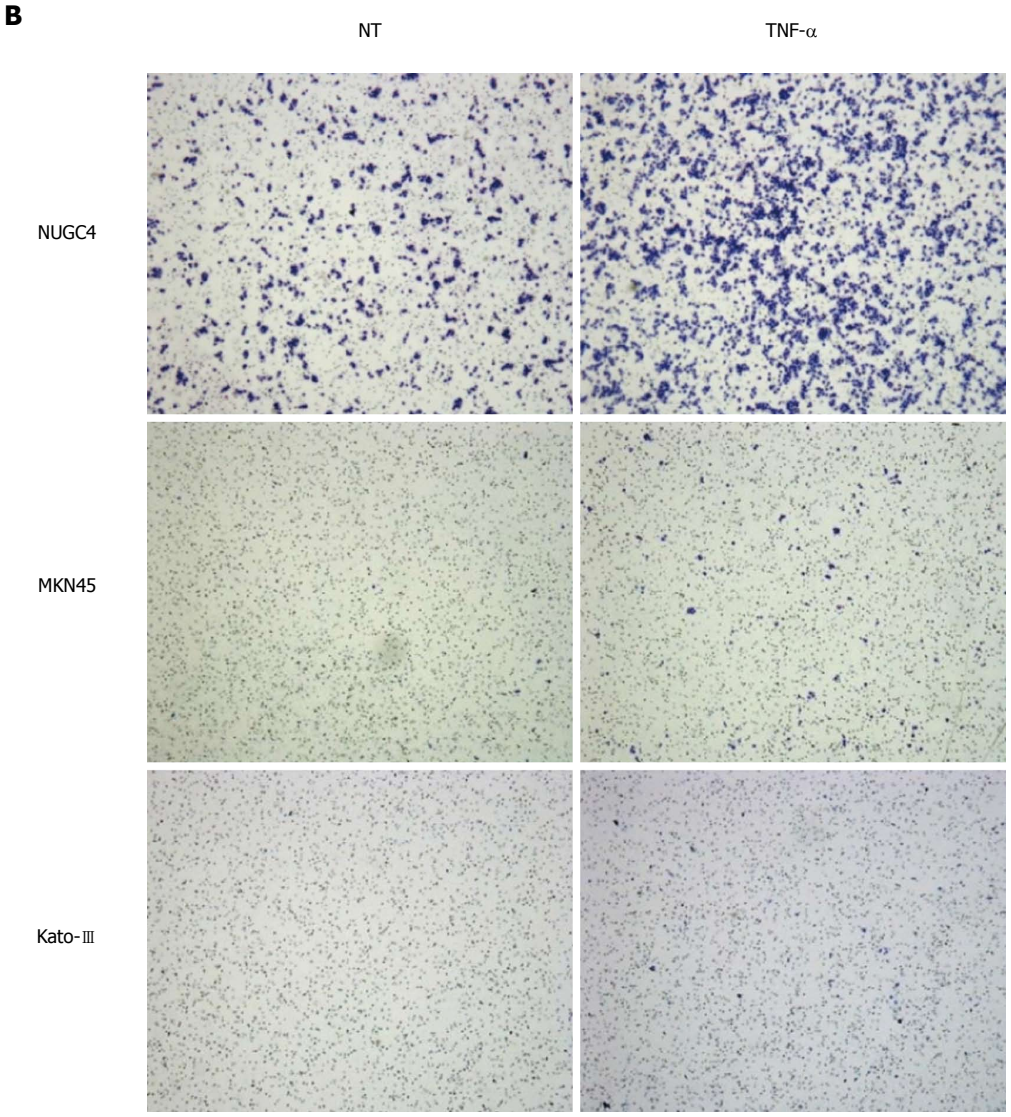
B
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Kato-Ⅲ

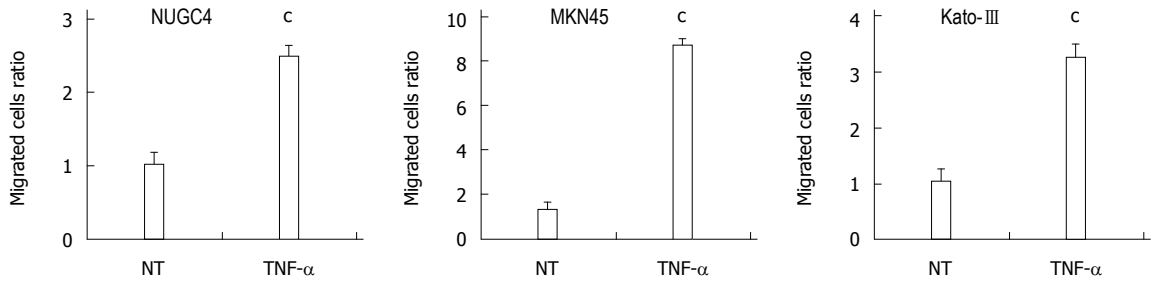

Figure 6 Tumor necrosis factor α induces cell migration in gastric cancer cells. A: Tumor necrosis factor α (TNF-α) stimulation (20 ng/mL, 24 h) significantly increased cell migration in MKN28 cells. Cell migration was determined by Boyden chamber assay. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA, NT; B: TNF-α stimulation (20 ng/mL, 48 h) significantly increased cell migration in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ. Cell migration was determined by Boyden chamber assay. Mean ± SE. $n = 3$. $^{c}P < 0.05$ *vs* control siRNA. NT: No treatment.

Table 1 Genes that displayed the greatest changes in their expressions in the claudin 1 small interfering RNA transfected MKN28 cells

Gene symbol	Gene ID	Gene name	Fold change
Up-regulated genes			
FGF6	NM_020996	Fibroblast growth factor 6	151.34
TM4SF20	NM_024795	Transmembrane 4 L six family member 20	72.03
B4GALNT2	NM_153446	Beta-1,4-N-acetyl-galactosaminyl transferase 2	47.30
SLC2A13	BC047507	Solute carrier family 2 (facilitated glucose transporter), member 13	43.02
HS3ST2	NM_006043	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	28.39
ZNF596	NM_001042416	Zinc finger protein 596	26.36
IL2RA	NM_000417	Interleukin 2 receptor, alpha	22.00
CCL2	NM_002982	Chemokine (C-C motif) ligand 2	18.05
SGOL2	BC048349	Shugoshin-like 2	17.64
SYT4	NM_020783	Synaptotagmin Ⅳ	17.29
FGF19	NM_005117	Fibroblast growth factor 19	16.44
ARID5B	NM_032199	AT rich interactive domain 5B	14.47
DEFB105B	NM_001040703	Defensin, beta 105B	14.34
DSG3	NM_001944	Desmoglein 3	12.99
RSPH1	NM_080860	Radial spoke head 1 homolog (Chlamydomonas)	12.82
COL5A2	NM_000393	Collagen, type Ⅴ, alpha 2	12.72
NRG2	NM_013982	Neuregulin 2	12.63
TMEM26	NM_178505	Transmembrane protein 26	12.22
CCL3L3	NM_001001437	Chemokine (C-C motif) ligand 3-like 3	11.54
OR51E1	NM_152430	Olfactory receptor, family 51, subfamily E, member 1	9.43
Down-regulated genes			
LY6G6F	NM_001003693	Lymphocyte antigen 6 complex, locus G6F	-332.04
WT1	NM_024426	Wilms tumor 1	-68.28
MADCAM1	NM_130760	Mucosal vascular addressin cell adhesion molecule 1	-60.84
SLC2A9	NM_001001290	Solute carrier family 2 (facilitated glucose transporter), member 9	-48.05
SULT4A1	NM_014351	Sulfotransferase family 4A, member 1	-39.30
SYNPR	NM_144642	Synaptoporin	-36.73
C10orf47	NM_153256	Chromosome 10 open reading frame 47	-29.96
MAS1L	NM_052967	MAS1 oncogene-like	-29.67
CLDN1	NM_021101	Claudin 1	-26.87
CBX1	NM_006807	Chromobox homolog 1	-26.37
MPPED2	NM_001145399	Metallophosphoesterase domain containing 2	-25.00
SCUBE2	NM_020974	Signal peptide, CUB domain, EGF-like 2	-22.13
CHI3L2	NM_001025199	Chitinase 3-like 2	-21.67
SLIT2	NM_004787	Slit homolog 2	-21.60
HFM1	NM_001017975	HFM1, ATP-dependent DNA helicase homolog	-20.56
CR1	NM_000651	Complement component (3b/4b) receptor 1	-18.04
PLA2G10	NM_003561	Phospholipase A2, group X	-16.70
GRIP2	NM_001080423	Glutamate receptor interacting protein 2	-16.45
XIST	NR_001564	X (inactive)-specific transcript	-15.79
TNF-SF10	NM_003810	Tumor necrosis factor (ligand) superfamily, member 10	-15.26

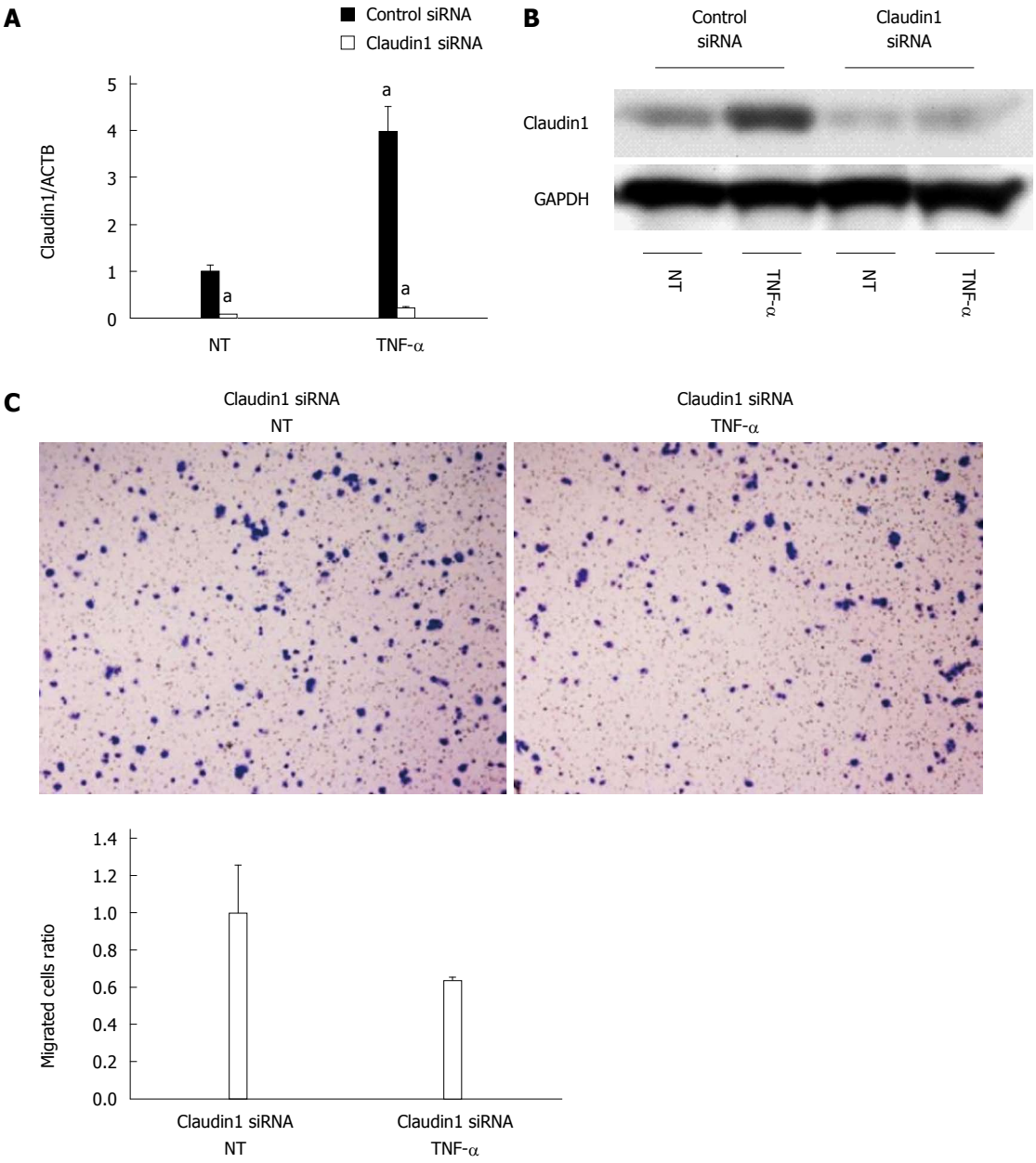

Figure 7 Claudin 1 is important for tumor necrosis factor α-induced cell migration in MKN28 cells. A: Claudin 1 siRNA effectively reduced both the basal and tumor necrosis factor α (TNF-α)-induced expression of claudin 1 mRNA. Mean ± SE. $n = 3$. $^{a}P < 0.05$ *vs* control siRNA, NT; B: Western blotting revealed that transfection of claudin 1 siRNA effectively reduced both the basal and TNF-α-induced expression of claudin 1 protein; C: TNF-α stimulation (20 ng/mL, 48 h) did not enhance cell migration in the claudin 1 siRNA transfected cells. Cell migration was determined by Boyden chamber assay. Mean ± SE. $n = 3$. NT: No treatment.

induced gene expression and cellular movement in human lung carcinoma A549 cells[21]. In the present study, our microarray data showed TNF- and NF-κB were the top-ranked upstream regulators related to claudin 1 in MKN28 cells. To determine the effect of TNF-α on claudin 1 expression in MKN28 cells, we analyzed protein levels of claudin 1, using western blotting. The basal expression level of claudin 1 was increased in a time-dependent manner at 24 and 48 h (Figure 5A). And, it was further increased by TNF-α treatment in MKN28 cells (Figure 5A). Similar trends that the expression level of claudin 1 was increased by TNF-α stimulation were found in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ (Figure 5B). Furthermore, results of immunofluorescent staining showed that TNF-α-induced claudin 1 expression was mainly detected in the cytoplasm of MKN28 cells (Figure 5C). TNF-α treatment increased cell migration in MKN28 cells (Figure 6A). Similar trends were found in several gastric cancer cell lines, including NUGC4, MKN45 and Kato-Ⅲ (Figure 6B).We then transfected MKN28 cells with claudin 1 siRNA and found that both the basal and TNF-α-induced expression of claudin 1 mRNA and protein were effectively reduced (Figure 7A, B). To examine TNF-α-induced gene expression, we performed microarray to determine gene expression profiles in cells treated with or without TNF-α and in the presence of claudin 1 siRNA or control siRNA. In control siRNA transfected MKN28 cells, TNF-α

Table 2 Top molecular and cellular functions, upstream regulators, and networks of claudin1 according to ingenuity pathway analysis

Name	*P* value	Molecules	Score
Top molecular and cellular functions			
Cellular movement	2.77×10^{-9}-1.01×10^{-2}	51	
Cell-to-cell signaling and interaction	7.24×10^{-7}-1.02×10^{-2}	55	
Molecular transport	1.23×10^{-6}-9.98×10^{-3}	51	
Small molecule biochemistry	1.23×10^{-6}-9.98×10^{-3}	55	
Cellular development	3.80×10^{-6}-9.50×10^{-3}	59	
Top upstream regulators			
TNF-RSF1A	1.80×10^{-7}		
TNF-	1.70×10^{-6}		
NF-κB (complex)	2.01×10^{-6}		
Escherichia coli B5 lipopolysaccharide	2.09×10^{-6}		
NF-κB1	3.23×10^{-6}		
Top networks			
Associated network functions			
Cellular assembly and organization, hair and skin development and function, cellular function and maintenance			33
Cell-to-cell signaling and interaction, cellular growth and proliferation, tissue morphology			32
Cellular movement, hematological system development and function, immune cell trafficking			31
Cardiovascular system development and function, organismal development, cellular movement			22
Hereditary disorder, respiratory disease, tissue development			22

TNF-: Tumor necrosis factor; NF: Nuclear factor.

stimulation changed expression of 169 genes, of which changes of 135 genes were not seen in claudin 1 siRNA transfected cells. For example, of the top 10 genes up-regulated by TNF-α, the fold of change was reduced in 7 of them, and all the top 10 genes down-regulated by TNF-α, the fold of changes was dramatically reduced in claudin 1 siRNA transfected MKN28 cells (Table 3). Moreover, TNF-α did not enhance cell migration in the claudin 1 siRNA transfected MKN28 cells (Figure 7C). These results show that claudin 1 has crucial role in mediating TNF-α-induced gene expression and migration in gastric cancer cells.

DISCUSSION

Recent reports have indicated that claudin proteins were up-regulated and mis-located in cancer cells[2]. Although there are several reports on the expression of claudin 1 in gastric cancer, no consensus has been reached about the relationship between claudin 1 expression and clinicopathological parameters[5,6,13,27]. Some reports have indicated that claudin 1 was highly expressed in intestinal subtype of adenocarcinomas[5,27], while others found that it was highly expressed in diffuse subtype of adenocarcinomas[6,13]. Several studies reported claudin 1 expression at invasive front of gastric carcinomas, which suggests that over expression of claudin 1 is related to carcinogenesis in invasive and metastatic gastric cancer[6,13,27]. The up-regulation of claudin 1 in colon cancer cells increased tumor growth and metastasis *in vivo*, whereas depletion of claudin 1 in metastatic colon cancer cells with siRNA inhibited cellular invasion[7]. Furthermore, over expression of claudin 1 increased cellular movement in melanoma[8], oral squamous cell carcinoma[9], hepatocellular carcinoma[10] and lung carcinoma cells[21]. In the present study, our results also showed that knocking down of claudin 1 decreased cell proliferation, cell migration and invasion, and increased apoptosis in gastric cancer cells, which suggests the importance of claudin 1 in the progression of gastric carcinomas.

Our data showed that "Cellular Movement" was the top-ranked molecular and cellular functions related to claudin 1 down-regulation according to Ingenuity Pathway Analysis in gastric cancer cells. MMP7 was found in the signal network related to cellular movement, strongly suggesting that the expression of claudin 1 may involve in migration, invasion and metastasis of gastric cancer. Furthermore, TNF- and NF-κB were the top-ranked upstream regulators related to claudin 1. Tumor necrosis factor superfamily member 10 (TNF-SF10), was significantly down-regulated by claudin 1 siRNA in MKN28 cells. These data show the important role of claudin 1 in TNF-α-induced cell migration.

It has been shown that TNF-α stimulation induced EMT in colonic organoids[14], renal carcinoma[28-30] and skin cells[31]. TJ and adherens junction proteins are usually down-regulated during the progression of EMT[15-20]. In this manner, claudin 1 expression is generally decreased by TNF-α stimulation, and the decreased protein expression leads to the increase in the paracellular permeability of epithelial cells[32,33]. On the other hand, TNF-α was reported to increase claudin 1 protein expression in pancreatic cancer cells[34] and airway smooth muscle cells[35]. Our recent report showed that TNF-α stimulation remarkably increased claudin 1 protein expression in the cytoplasm in A549 cells, and that depletion of claudin

Table 3 Top 10 up or down-regulated genes induced by tumor necrosis factor α in control small interfering RNA or claudin 1 small interfering RNA transfected MKN28 cells

Gene symbol	Gene ID	Gene name	Control siRNA	Claudin1 siRNA
			Fold change	Fold change
Up-regulated genes				
IL6	NM_000600	Interleukin 6	11.16	7.16
MMP9	NM_004994	Matrix metallopeptidase 9	10.49	18.13
BMP2	NM_001200	Bone morphogenetic protein 2	10.12	5.62
VSTM1	NM_198481	V-set and transmembrane domain containing 1	9.83	2.53
CLEC1A	NM_016511	C-type lectin domain family 1, member A	9.71	-1.22
CXCL10	NM_001565	Chemokine (C-X-C motif) ligand 10	9.33	1.82
IL2RG	NM_000206	Interleukin 2 receptor, gamma	9.14	15.56
TFPI	NM_001032281	Tissue factor pathway inhibitor	8.98	11.79
KLHDC7B	NM_138433	Kelch domain containing 7B	8.90	1.79
CCL5	NM_002985	Chemokine (C-C motif) ligand 5	8.85	7.84
Down-regulated genes				
LY6G6F	NM_001003693	Lymphocyte antigen 6 complex, locus G6F	-311.81	1.05
RFTN2	NM_144629	Raftlin family member 2	-291.41	1.39
WT1	NM_024426	Wilms tumor 1	-75.96	1.05
MADCAM1	NM_130760	Mucosal vascular addressin cell adhesion molecule 1	-67.39	1.06
SLC2A9	NM_001001290	Solute carrier family 2, member 9	-49.12	-1.21
C10orf47	NM_153256	Chromosome 10 open reading frame 47	-44.11	1.85
SYNPR	NM_144642	Synaptoporin	-40.64	1.08
GGT7	NM_178026	Gamma-glutamyltransferase 7	-40.33	1.24
LRRC36	NM_018296	Leucine rich repeat containing 36	-38.25	1.06
MAS1L	NM_052967	MAS1 oncogene-like	-32.62	1.07

1 inhibited the TNF-α-induced gene expression and cell migration[21]. Similarly, in the present study, TNF-α induced over expression of claudin 1 in the cytoplasm in gastric carcinoma MKN28 cells, and knocking down of claudin 1 blocked the TNF-α-induced gene expression and cellular movement in human gastric cancer cells. Generally, claudin 1 participates in cell-to-cell adhesion as TJ proteins, and its down-regulation may promote cell migration. However, our findings showed claudin 1 induced by TNF-α is mainly in cytoplasm, and regulates TNF-α-induced gene expression[21]. In addition, many of these claudin 1 dependent genes are related to cellular movement, suggesting that claudin 1 mediates TNF-α-initiated cell migration with various mechanisms[21].

In conclusion, we found that claudin 1 played a role in the proliferation, apoptosis, cell migration and invasion in gastric carcinoma cells, suggesting the importance of claudin 1 expression in the progression of gastric carcinomas. Our microarray results also suggest that claudin 1 has marked effects on the expression of genes related to cellular movement. Furthermore, we showed that TNF-α induces the gene expression of claudin 1 in gastric carcinoma cells, and the latter acts as the signal mediator to regulate gene expression and cellular movement. A deeper understanding of this pathway may serve as a mean to establish a new therapeutic target for gastric carcinoma.

COMMENTS

Background

Recent reports have indicated that claudin proteins were up-regulated and mislocated in cancer cells, and influenced the biological behavior of tumor progression. Although there are several reports on the expression of claudin 1 in gastric cancer, no consensus has been reached about the relationship between claudin 1 expression and clinicopathological features.

Research frontiers

The authors recently found that the expression of claudin 1 was increased in response to tumor necrosis factor alpha (TNF-α) stimulation, and that claudin 1 played an important role in TNF-α-induced gene expression and cellular movement in human lung carcinoma A549 cells. The objectives of the present research were to investigate the role of claudin 1 in the control of genes involved in cell migration and TNF-α-induced gene expression in human gastric adenocarcinoma cells.

Innovations and breakthroughs

The authors showed that the knockdown of claudin 1 significantly inhibited cell migration and invasion in gastric cancer cells. Microarray analyses showed that down-regulation of claudin 1changed the expression levels of many genes related to cellular movement and TNF-α signal. They found that TNF-α stimulation induced the gene expression of claudin 1 in gastric carcinoma cells, and the latter acted as the signal mediator to regulate gene expression and migration.

Applications

The results of the present study suggest that claudin 1 acts as a crucial signal mediator in TNF-α induced gene expression and cell migration in gastric carcinoma cells. A deeper understanding of these cellular processes may be helpful in establishing new therapeutic strategies for gastric cancer.

Terminology

Claudin proteins play an essential role in the function of TJ, and 24 subtypes of the claudin have been identified. They interact with each other through homo- and heterophilic interactions and are crucial for the maintenance of cellular polarity of epithelial cells.

Peer review

This is a good descriptive study in which the role of claudin 1 in the regulation of genes involved cell migration and TNF-α-induced gene expression in human gastric cancer cells. They reported that claudin 1 knock down significantly inhibited cell migration and invasion in gastric carcinoma cells. And the depletion of claudin 1 changed the expression level of TNF-α signal. The results are interesting and meaningful for further understand the role of claudin 1 on cancer development.

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ORIGINAL ARTICLE

Study of pruritus in chronic hepatitis C patients

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Abstract

AIM: To investigate the occurrence and severity of pruritus in chronic hepatitis C patients treated with or without interferon (IFN) therapy.

METHODS: A total of 89 patients with chronic hepatitis C and 55 control (non-hepatitis) patients were asked to rate their experience of diurnal and nocturnal pruritus in the preceding week using a visual analogue scale (VAS) and a five-point scale, respectively. Blood samples were taken and serum thymus and activation-regulated chemokine (TARC) levels were measured by enzyme-linked immunosorbent assay.

RESULTS: A significantly greater proportion of chronic hepatitis C patients experienced nocturnal pruritus compared with control (58.4% *vs* 5.5%, $P < 0.0001$). Chronic hepatitis C patients also had more severe pruritus compared with control patients, indicated by the higher mean VAS scores in both the IFN-treated and non-IFN-treated groups. In particular, patients who received combined peginterferon alfa-2b and ribavirin had significantly higher mean VAS scores than those receiving peginterferon alfa-2a or no IFN treatment. Serum TARC levels did not correlate with pruritus scores, and no significant differences in TARC levels were observed between the IFN-treated and non-IFN-treated groups.

CONCLUSION: Patients with chronic hepatitis C experience pruritus more than those without. Serum TARC levels do not correlate with pruritus severity in chronic hepatitis C patients.

Key words: Hepatitis C; Pruritus; Peginterferon; Ribavirin; Thymus and activation-regulated chemokine

Core tip: This is the first paper to evaluate the occurrence and severity of pruritus in chronic hepatitis C patients and to examine the relationship between pruritus and interferon therapy. We found that patients with chronic hepatitis C experience pruritus more than those without chronic hepatitis C.

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INTRODUCTION

Little is known about pruritus associated with interferon (IFN) therapy for chronic hepatitis C[1]. Chronic hepatitis C patients also experience pruritus in the absence of IFN therapy. Serum thymus and activation-regulated chemokine (TARC) levels reflect the activity of atopic dermatitis[2], but the relationship between TARC levels and pruritus in chronic hepatitis C patients has not been studied.

The purpose of this study was to examine the occurrence and severity of pruritus in chronic hepatitis C compared with other gastrointestinal disorders, and to exam-

ine the relationship between pruritus and IFN therapy. We also examined the relationship between serum TARC levels and pruritus in chronic hepatitis C patients.

MATERIALS AND METHODS

Patients

This study was approved by the Ethics Committee of Dokkyo Medical University Koshigaya Hospital and written informed consent was obtained from all participants. This study conformed to the ethical guidelines of the 2008 Declaration of Helsinki. Subjects were 89 chronic hepatitis C patients who were treated in the Department of Gastroenterology of Dokkyo Medical University Koshigaya Hospital between October 2010 and September 2012 (chronic hepatitis C group). This group comprised 44 men and 45 women with a mean age of 57.8 ± 10.6 years (range: 27-78 years). Of these, 54 patients were receiving IFN therapy (IFN group), while the remaining 35 patients did not receive IFN therapy (non-IFN group). Of those treated with IFN, 10 patients were given peginterferon alfa-2a (PEG-IFN α2a) monotherapy (α2a group), while 44 received combined peginterferon alfa-2b (PEG-IFN α2b) and ribavirin (RBV) therapy (α2b + RBV group).

The control group comprised 55 patients treated in our department for non-hepatic disorders during the same period. The group comprised 30 men and 25 women with a mean age of 58.7 ± 13.6 years (range: 24-74 years). Ideally, the control group should have comprised healthy individuals; however, such subjects were not available as the study population was selected from patients in our hospital. There was no statistical difference in the age or sex between these patients and those with chronic hepatitis C. Control patients had the following gastrointestinal disorders: reflux esophagitis (n = 23), atrophic gastritis (n = 15), gastrointestinal ulcers (n = 6), gallbladder polyps (n = 4), and other gastrointestinal disorders (n = 7). Patients with a history of atopic dermatitis or other skin conditions, malignant tumors, or those who used anti-allergic mediation or oral/topical steroids were excluded from the study.

Evaluation of pruritus

Pruritus was defined as generalized itching in the absence of a rash or erythema; localized itching at IFN injection sites, insect bites or hives were not included in the analysis. Two types of patient-based instruments were used to assess pruritus. In the first instrument, nocturnal pruritus severity was assessed using a scale described by Kawashima *et al*[3] in which nocturnal pruritus in the preceding week was rated on a five-point scale from 0 (none) to 5 (very severe) (Table 1). In the second instrument, the diurnal pruritus severity in the preceding week was recorded on a visual analogue scale (VAS) and converted to a numerical score (Figure 1). For patients undergoing IFN therapy, assessment of nocturnal and diurnal pruritus severity was conducted during the 4- to 8-wk period after initiation of IFN therapy.

Table 1 Scoring of nocturnal pruritus

Score	Nocturnal pruritus
4	Very severe, interfering with sleep
3	Severe, very annoying, substantially interfering with sleep
2	Moderate, annoying and troublesome, may interfere with sleep
1	Mild, not annoying or interfering with sleep
0	None

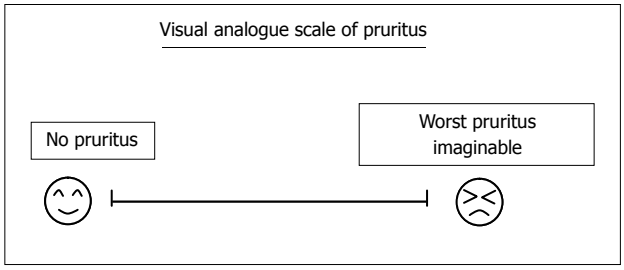

Figure 1 Visual analogue scale. Patients marked a point on the scale corresponding to the severity of pruritus experienced in the preceding week. The scale was 10 cm in length with "worst possible pruritus" on the right-hand end of the line and "no pruritus" on the left-hand end of the line.

Measurement of serum TARC and other laboratory parameters

Blood was taken from patients in the chronic hepatitis C group at the time when the pruritus assessment was performed. The serum was stored at -40 ℃, and TARC levels were measured by enzyme-linked immunosorbent assay. The following laboratory parameters were also measured in the chronic hepatitis C group: alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum γ-glutamyltransferase (GGT), total bilirubin and albumin, platelet count, and prothrombin activity.

Statistical analysis

Pruritus and VAS scores, TARC levels, and laboratory parameter values are expressed as mean ± SE. The Mann-Whitney U test was used to compare differences between two groups, and the Kruskal-Wallis test was used to compare multiple groups. $P < 0.05$ was regarded as statistically significant.

RESULTS

Relationship between nocturnal pruritus score and visual analogue scale

Figure 2 shows the nocturnal pruritus scores of the chronic hepatitis C and control groups. Patients in the chronic hepatitis C group had the following scores: 37 had a score of 0; 15 had a score of 1; 26 had a score of 2; 11 had a score 3; and none of them had a score of 4. Thus, 52 of 89 patients (58.4%) in the chronic hepatitis C group had a nocturnal pruritus score ⩾ 1. Patients in the control group had the following scores: 52 patients had a

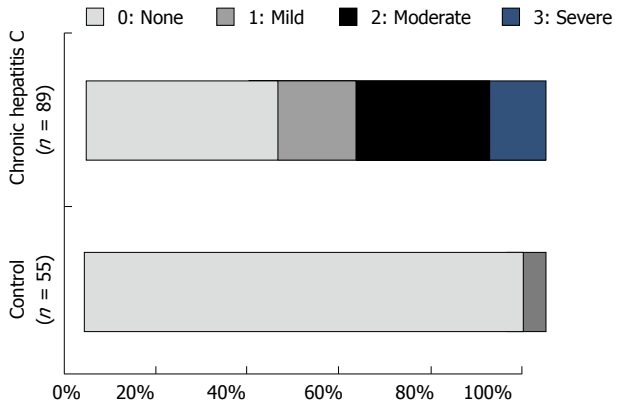

Figure 2 Pruritus scores of chronic hepatitis C and control groups. A total of 52 of 89 patients (58.4%) in the chronic hepatitis C group had nocturnal pruritus scores ⩾ 1. Only 3 of 52 patients (5.5%) in the control group had pruritus; the proportion of patients with pruritus in the control group was significantly lower than that in the chronic hepatitis C group ($P < 0.0001$).

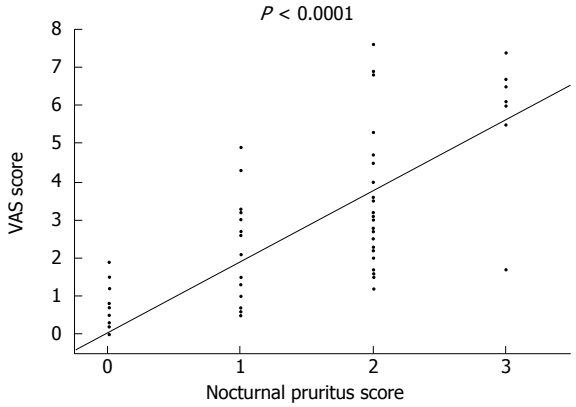

Figure 3 Relationship between nocturnal pruritus scores and visual analogue scale scores of all 144 subjects. Nocturnal pruritus scores of 0, 1, 2, and 3 correspond to VAS scores of 0.09 ± 0.04 cm, 2.32 ± 0.54 cm, 3.50 ± 0.37 cm, and 6.23 ± 0.55 cm, respectively, with a good correlation between the pruritus score and VAS score ($P < 0.0001$). VAS: Visual analogue scale.

Table 2 Clinical characteristics of chronic hepatitis C patients treated with or without interferon

	IFN (-), *n* = 35	IFN (+), *n* = 54	*P* value
Age	60.0 ± 12.3	56.4 ± 9.2	0.1112
Sex	M:17; F:18	M:27; F:27	0.6351
Duration of IFN therapy (wk)	NA	22.3 ± 17.4	NA
HCV RNA (Log IU/mL)	6.0 ± 1.3	1.2 ± 2.0	< 0.0001
ALT (U/L)	60.5 ± 52.6	33.4 ± 26.2	0.0017
ALP (U/L)	307.0 ± 108.0	265.2 ± 77.2	0.0360
GGT (U/L)	48.9 ± 37.41	45.4 ± 44.3	0.6981
T-Bil (mg/dL)	0.86 ± 0.28	0.85 ± 0.44	0.9323
Alb (g/dL)	4.19 ± 0.40	4.15 ± 0.44	0.6315
PLT (× 10^4/μL)	14.5 ± 5.1	13.1 ± 6.9	0.2938
PT	84.2% ± 7.5%	86.0 ± 10.1	0.7966

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: Serum γ-glutamyltransferase; T-Bil: Total bilirubin; Alb: Albumin; PLT: Platelets; PT: Prothrombin activity; NA: Not applicable.

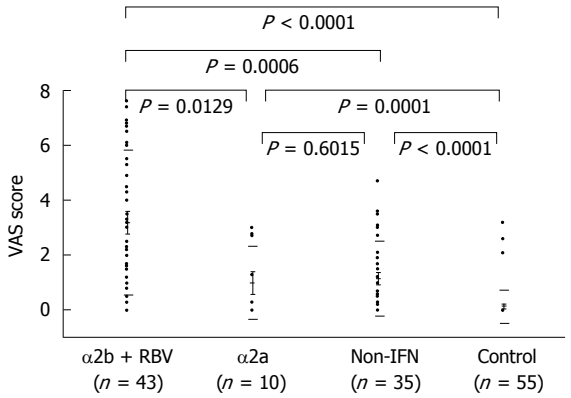

Figure 4 Visual analogue scale scores of chronic hepatitis C and control groups. With respect to chronic hepatitis C patients, the non-IFN group, α2a group, and α2b + RBV group had VAS scores of 1.13 ± 0.29, 1.01 ± 0.56, and 3.20 ± 0.27, respectively, all of which were significantly higher than that of the control group (0.32 ± 0.26). The VAS score was significantly different between the non-IFN group and α2b + RBV group, and between the α2a group and α2b + RBV group, but the VAS score was not significantly different between the non-IFN group and α2a group. IFN: Interferon; VAS: Visual analogue scale; RBV: Ribavirin.

score of 0; 3 patients had a score of 1; and none of them had a score ⩾ 2. Thus, only 3 of 55 patients (5.5%) in the control group had pruritus; the proportion of patients with pruritus in the control group was significantly lower than that in the chronic hepatitis C group ($P < 0.0001$). Figure 3 shows the relationship between nocturnal pruritus scores and VAS scores of the 144 subjects. Nocturnal pruritus scores of 0, 1, 2, and 3 corresponded to VAS scores of 0.09 ± 0.04 cm, 2.32 ± 0.54 cm, 3.50 ± 0.37 cm, and 6.23 ± 0.55 cm, respectively, with a good correlation between the pruritus score and VAS score ($P < 0.0001$). Based on this result, we used the VAS score to evaluate pruritus.

Association between pruritus and IFN therapy in chronic hepatitis C

Table 2 shows the characteristics of chronic hepatitis C patients treated with or without IFN. No significant difference in the age or sex between the two groups was noted. The mean duration of IFN treatment was 22.3 wk, and HCV RNA was significantly lower in the IFN group than the non-IFN group. ALT and ALP were significantly higher in the non-IFN group than the IFN group, but other clinical parameters were not significantly different.

Figure 4 shows the VAS scores for the chronic hepatitis C and control groups. The VAS score of the control group was 0.32 ± 0.26. With respect to chronic hepatitis C patients, the non-IFN group, α2a group, and α2b + RBV group had VAS scores of 1.13 ± 0.29, 1.01 ± 0.56, and 3.20 ± 0.27, respectively, all of which were significantly higher than that of the control group. The VAS score was significantly different between the non-IFN group and α2b + RBV group, and between the α2a group and α2b + RBV group, but the VAS score was not significantly different between the non-IFN group and

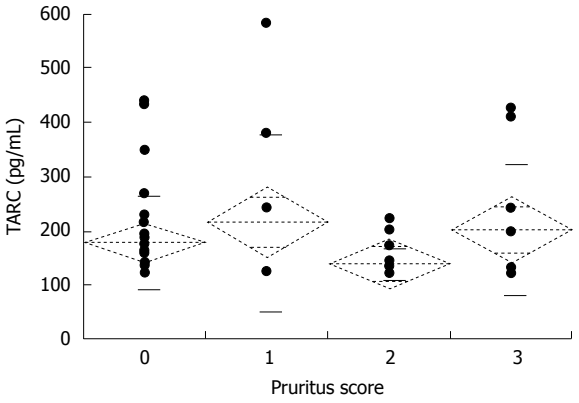

Figure 5 Association between pruritus score and serum thymus and activation-regulated chemokine level. The mean TARC levels for the pruritus scores 0, 1, 2, and 3 were 180.0 ± 16.4 pg/mL, 217.8 ± 54.3 pg/mL, 140.8 ± 7.0 pg/mL, and 203.7 ± 38.0 pg/mL, respectively. The pruritus score did not correlate with TARC levels. TARC: Thymus and activation-regulated chemokine.

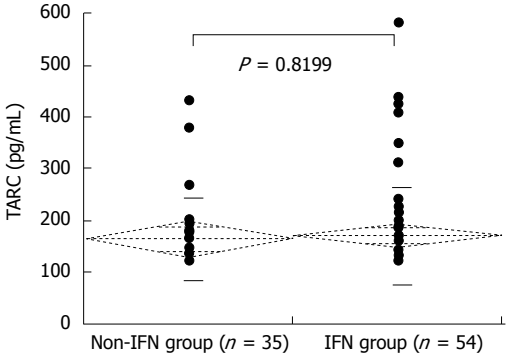

Figure 6 Interferon treatment and serum thymus and activation-regulated chemokine levels in chronic hepatitis C. The mean serum TARC levels of the non-IFN and IFN groups were 166.8 ± 79.1 pg/ml and 173.3 ± 92.1 pg/mL, respectively; the difference in TARC levels between the two groups was not significant (P = 0.8199). TARC: Thymus and activation-regulated chemokine; IFN: Interferon.

α2a group.

Association between pruritus and serum TARC levels in chronic hepatitis C

Figure 5 shows the nocturnal pruritus scores and serum TARC levels of the 89 chronic hepatitis C patients. The mean TARC levels for the pruritus scores 0, 1, 2, and 3 were 180.0 ± 16.4 pg/mL, 217.8 ± 54.3 pg/mL, 140.8 ± 7.0 pg/mL, and 203.7 ± 38.0 pg/mL, respectively. The pruritus score did not correlate with TARC levels. The mean serum TARC levels of the non-IFN and IFN groups were 166.8 ± 79.1 pg/mL and 173.3 ± 92.1 pg/mL, respectively; the difference in TARC levels between the two groups was not significant (Figure 6). The mean serum TARC levels of the α2a and α2b + RBV groups were 165.1 ± 30.9 pg/mL and 174.7 ± 12.8 pg/mL, respectively; the difference in TARC levels between the two groups was not significant (Figure 7).

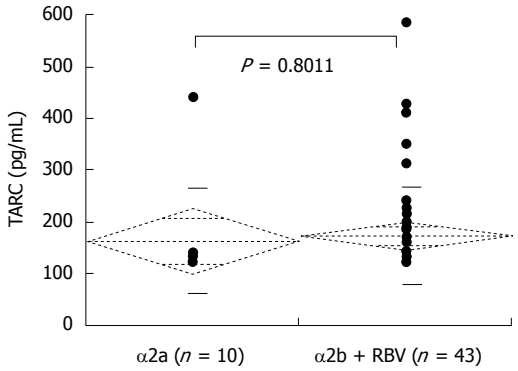

Figure 7 Serum thymus and activation-regulated chemokine level by treatment method in chronic hepatitis C patients. The mean serum TARC levels of the α2a and α2b + RBV groups were 165.1 ± 30.9 pg/mL and 174.7 ± 12.8 pg/mL, respectively; the difference in TARC levels between the two groups was not significant (P = 0.8011). TARC: Thymus and activation-regulated chemokine; RBV: Ribavirin.

DISCUSSION

Pruritus occurs in 2.5%-23% of patients with chronic hepatitis C[4-7]. The cause of pruritus in chronic hepatitis C is not well understood, although it has been associated with liver fibrosis, bile duct lesions and cholestasis[8-11]. In addition, hepatitis C may cause a primary skin disorder with pruritus as a symptom[5]. In this study, 58.4% of chronic hepatitis C patients experienced pruritus, which was higher than the 5.5% of control patients. There was no difference in the age or sex between the two groups, and patients were enrolled in the study at the same time, so we do not expect any differences in environmental factors such as temperature and humidity that might have influenced the results. Forty-four percent of chronic hepatitis C patients who did not receive IFN had pruritus, which was significantly higher than that seen in control patients (data not shown).

Adverse skin reactions and pruritus during IFN treatment for chronic hepatitis C may be caused by either immune changes or an excessive immune reaction[12,13]. Peginterferon induces injection site reactions in 36%-59% of cases[14,15] and can sometimes cause generalized dermatitis[13,16,17]. In our study of chronic hepatitis C patients, we only assessed the presence of pruritus and excluded injection site reactions and atopic dermatitis from the analysis.

The severity of pruritus as indicated by the VAS score was examined in subgroups of chronic hepatitis C patients. No significant difference in the VAS score between the non-IFN group and the α2a group was observed. Significant differences in VAS scores were seen between the non-IFN and α2b + RBV groups (P = 0.0006), and between the α2a and α2b + RBV groups (P = 0.0129). PEG-IFN α2a has a branched 40-kDa PEG chain, whereas PEG-IFN α2b has a linear 12-kDa PEG chain. Although PEG-IFN α2a and PEG-IFN α2b are expected to have similar side effect profiles[18], the differ-

ence in molecular weight might affect the development of pruritus due to these agents, but this is difficult to prove experimentally. Comparison between PEG-IFN α2a and PEG-IFN α2b monotherapies would have been preferable; however, this was not possible because the Japanese health insurance system stipulates that PEG-IFN α2b must be administered with RBV.

RBV itself has been reported to cause pruritus[13,19]. When combined with PEG-IFN, a synergistic effect may result in a delayed-type hypersensitivity reaction, with a shift in the helper T (Th) cell balance towards a Th1-dominant response due to the influence of IFN. This may explain the higher prevalence of pruritus in the α2b + RBV group[20].

Chemokines serve as chemotactic factors for immune cells particularly leukocytes. Chemokines are categorized into four types-namely, C, CC, CXC and CX_3C-depending on the position of cysteine in their amino acid chains. The normal level of TARC (a CC chemokine) in healthy adults is ⩽ 450 pg/mL. In patients with moderate atopic dermatitis, the serum TARC level is ⩾ 750 pg/mL and reflects the disease activity of this condition[2]. One study reported that HCV-induced TARC expression attracted Treg cells to the liver, and TARC expression was normalized in patients with a sustained virological response after IFN therapy[21]. Based on these results, we selected TARC as a marker for pruritus in hepatitis C patients in the present study. Although the development of dermatitis in atopic patients treated with IFN for chronic hepatitis C has been reported[22], our study is the first to investigate the relationship between TARC levels and pruritus in chronic hepatitis C patients. Our findings revealed a lack of correlation between serum TARC levels and pruritus scores in the 89 patients with chronic hepatitis C. Also, no significant differences in the serum TARC levels between the non-IFN and IFN groups, or between the α2a and α2b + RBV groups, were noted. Notably, 3 of 54 patients (5.6%) treated with IFN had pruritus and skin lesions such as erythema, eczema, and erosion at non-injection sites (data not shown). Although the VAS score was higher in these patients, only one patient had an elevated TARC level. Despite the unknown association between pruritus in chronic hepatitis C patients and atopic dermatitis, our results suggest that serum TARC levels do not reflect the severity of skin symptoms in chronic hepatitis C patients during IFN treatment.

Patients with chronic hepatitis C experience pruritus more than those without chronic hepatitis C, and pruritus is particularly worse in patients undergoing combined PEG-IFN α2b and RBV therapy. Serum TARC levels do not correlate with pruritus severity in chronic hepatitis C patients.

COMMENTS

Background

Little is known about pruritus associated with interferon (IFN) therapy for chronic hepatitis C. Chronic hepatitis C patients also experience pruritus in the absence of IFN therapy. Serum thymus and activation-regulated chemokine (TARC) levels reflect the activity of atopic dermatitis, but the relationship between TARC levels and pruritus in chronic hepatitis C patients has not been studied.

Research frontiers

The normal level of TARC (a CC chemokine) in healthy adults is ⩽ 450 pg/mL. In patients with moderate atopic dermatitis, the serum TARC level is ⩾ 750 pg/mL and reflects the disease activity of this condition. One study reported that HCV-induced TARC expression attracted Treg cells to the liver, and TARC expression was normalized in patients with a sustained virological response after IFN therapy. Based on these results, we selected TARC as a marker for pruritus in hepatitis C patients in the present study. Although the development of dermatitis in atopic patients treated with IFN for chronic hepatitis C has been reported, our study is the first to investigate the relationship between TARC levels and pruritus in chronic hepatitis C patients.

Innovations and breakthroughs

In this study, 58.4% of chronic hepatitis C patients experienced pruritus, which was higher than the 5.5% of control patients. The severity of pruritus as indicated by the VAS score was examined in subgroups of chronic hepatitis C patients. No significant difference in the VAS score between the non-IFN group and the α2a group was observed. Significant differences in VAS scores were seen between the non-IFN and α2b + RBV groups (P = 0.0006), and between the α2a and α2b + RBV groups (P = 0.0129). This paper revealed a lack of correlation between serum TARC levels and pruritus scores in the 89 patients with chronic hepatitis C. Also, no significant differences in the serum TARC levels between the non-IFN and IFN groups, or between the α2a and α2b + RBV groups, were noted. Patients with chronic hepatitis C experience pruritus more than those without chronic hepatitis C, and pruritus is particularly worse in patients undergoing combined PEG-IFN α2b and RBV therapy. Serum TARC levels do not correlate with pruritus severity in chronic hepatitis C patients.

Applications

The study results showed the actual situation of pruritus in the hepatitis C patient.

Terminology

Chemokines serve as chemotactic factors for immune cells particularly leukocytes. Chemokines are categorized into four types-namely, C, CC, CXC and CX_3C-depending on the position of cysteine in their amino acid chains. TARC is CC chemokine.

Peer review

The authors of this manuscript discuss pruritus in chronic hepatitis C and tried to correlate this symptom with serum TARC levels. Unfortunately however, TARC levels were not correlated with pruritus in chronic hepatitis C with or without IFN treatment. They found a strong correlation between pruritus and Peg-IFNα2b + RBV combination therapy. The obtained results in this study are in accordance with our experience in daily clinical practice.

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ORIGINAL ARTICLE

Differential gene expression profiling of gastric intraepithelial neoplasia and early-stage adenocarcinoma

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Abstract

AIM: To investigate the differentiated whole genome expression profiling of gastric high- and low-grade intraepithelial neoplasia and early-stage adenocarcinoma.

METHODS: Gastric specimens from an upper magnifying chromoendoscopic targeted biopsy were collected from March 2010 to May 2013. Whole genome expression profiling was performed on 19 low-grade intraepithelial neoplasia (LGIN), 20 high-grade intraepithelial neoplasia (HGIN), 19 early-stage adenocarcinoma (EGC), and 19 chronic gastritis tissue samples using Agilent 4 × 44K Whole Human Genome microarrays. Differentially expressed genes between different types of lesions were identified using an unpaired *t*-test and corrected with the Benjamini and Hochberg false discovery rate algorithm. A gene ontology (GO) enrichment analysis was performed using the GeneSpring software GX 12.6. The differentially expressed gene was verified using a real-time TaqMan® PCR assay with independent tissue samples, including 26 LGIN, 15 HGIN, 14 EGC, and 20 chronic gastritis. The expression of G_0S_2 were further validated by immunohistochemical staining (IHC) in 24 LGIN, 40 HGIN, 30 EGC and 61 chronic gastritis specimens.

RESULTS: The gene expression patterns of LGIN and HGIN tissues were distinct. There were 2521 significantly differentially expressed transcripts in HGIN, with 951 upregulated and 1570 downregulated. A GO enrichment analysis demonstrated that the most striking overexpressed transcripts in HGIN compared with LGIN were in the category of metabolism, defense response, and nuclear factor κB (NF-κB) cascade. While the vast majority of transcripts had barely altered expression in HGIN and EGC tissues, only 38 transcripts were upregulated in EGC. A GO enrichment analysis revealed that the alterations of the immune response were most prominent in the progression from HGIN to EGC. It is worth noting that, compared with LGIN, 289 transcripts

were expressed at higher levels both in HGIN and EGC. A characteristic gene, G_0/G_1 switch 2 (G_0S_2) was one of the 289 transcripts and related to metabolism, the immune response, and the NF-κB cascade, and its expression was validated in independent samples through real-time TaqMan® PCR and immunohistochemical staining. In real-time PCR analysis, the expression of G_0S_2 was elevated both in HGIN and EGC compared with that in LGIN ($P < 0.01$ and $P < 0.001$, respectively). In IHC analysis, G_0S_2 immunoreactivity was detected in the cytoplasmic of neoplastic cells, but was undetectable in chronic gastritis cells. The G_0S_2 expression in HGIN was higher than that of LGIN ($P = 0.012$, $\chi^2 = 6.28$) and EGC ($P = 0.008$, $\chi^2 = 6.94$).

CONCLUSION: A clear biological distinction between gastric high- and low-grade intraepithelial neoplasia was identified, and provides molecular evidence for clinical application.

Key words: Gastric early-stage adenocarcinoma; High- and low-grade intraepithelial neoplasia; G_0/G_1 switch 2; Whole genome expression microarray; Quantitative real-time PCR; Immunohistochemical staining

Core tip: This is the first study to perform a comprehensive detection of the gene expression profiling of gastric low-grade and high-grade intraepithelial neoplasia and early-stage adenocarcinoma. This study collected precise samples and reports a clear distinction of gene expression profiles between gastric low-grade and high-grade intraepithelial neoplasia, thus providing molecular evidence for their different clinical application. The characteristic upregulated genes during gastric early carcinogenesis were involved in metabolism and the immune response and nuclear factor-κB pathway, whose expression was validated in independent samples through real-time TaqMan® polymerase chain reaction and immunohistochemical staining.

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INTRODUCTION

Gastric cancer is the fourth most prevalent malignant disease and the second most common cause of cancer-related deaths worldwide. Early gastric cancer (EGC) is confined to the mucosa and submucosa of the stomach with or without lymph node metastasis. The 5-year survival rates between advanced-stage and early-stage gastric cancer are extremely different (10% and 90%, respectively)[1]. Unlike advanced-stage patients, patients with early-stage gastric cancer and precancerous lesions usually have no symptoms. Precancerous lesions are histological abnormalities and are more likely to occur with cancer. According to the WHO classification of tumors of the digestive system[2], the terms low-grade intraepithelial neoplasia/dysplasia (LGIN) and high-grade intraepithelial neoplasia/dysplasia (HGIN) are recommended for gastric precancerous lesions. Enhanced endoscopy increases the detection of asymptomatic gastric cancer or precancerous lesions for early intervention. Though they have a high incidence, Japan and South Korea have a low mortality-to-incidence ratio (0.43/0.35) that benefits from a population-based screening program[3]. Therefore, early detection and an appropriate diagnosis of gastric intraepithelial neoplasia and early-stage cancer are associated with improved outcomes for patients. Furthermore, it is important to understand more about these stages both clinically and biologically.

In the Correa cascade of multi-step gastric carcinogenesis[4], an inflammation-metaplasia-dysplasia-carcinoma sequence indicates that dysplasia may be a critical point for malignant transformation. A cohort study demonstrated that 24.9% of patients with severe dysplasia and 2.1% of patients with mild-to-moderate dysplasia received a diagnosis of gastric cancer within 1 year of follow-up after the initial diagnosis[5]. Most patients harboring lesions that are classified as high-grade dysplasia are at high risk of either synchronous invasive carcinoma or its rapid development[6]. Based on the potential transition between and morphological similarity of dysplasia and carcinoma, the hypothesis that they are biologically related is reasonable. Previous studies of the genomic copy number aberration of gastric precancerous lesions and carcinoma *in situ* (CIS) have provided the most prominent 8q gain, which was detected most frequently in both HGIN and CIS but was undetected in LGIN using array comparative genomic hybridization[7]. Therefore, evidence has shown that molecular variations in gastric carcinogenesis have already appeared in precancerous lesions or EGC.

According to the revised Vienna classification of gastrointestinal epithelial neoplasia, the clinical management of endoscopic follow-up is recommended for category 3 (LGIN), while endoscopic or surgical local resection is recommended for category 4 (HGIN). LGIN and HGIN apparently have different clinicopathological characteristics; however, little is known about their biological characteristics. Previous gene expression profiling studies on gastric precancerous lesions did not detail the differences between LGIN and HGIN.

In this study, the gene expression profiling of gastric high- and low-grade intraepithelial neoplasia and early-stage adenocarcinoma were investigated to explore the molecular alterations in the malignant progression of gastric neoplasia. A clear distinction of the gene expression profiles between HGIN and LGIN were identified, thus providing molecular evidence for their different clinical relevance. The microarray data were validated by quantitative real-time polymerase chain reaction (PCR) in an

independent group of patients, and followed by immunohistochemical (IHC) staining. Interestingly, characteristic upregulated genes during gastric early carcinogenesis were involved in metabolism and the immune response and the nuclear factor κB (NF-κB) pathway.

MATERIALS AND METHODS

Patients and frozen tissue samples

Subjects were recruited from Peking Union Medical College Hospital (PUMCH) and Qinghai Provincial People's Hospital, and provided 137 samples and 15 samples, respectively, between March 2010 and May 2013. Gastric specimens from an upper magnifying chromoendoscopic targeted biopsy were collected. The samples used for pathological diagnosis and for this experiment in each patient were very similar. According to the WHO Classification of Tumors of the Digestive System, the samples can be grouped into 4 categories: LGIN (8148/0), HGIN (8148/2), EGC (8140/3), and the chronic gastritis group.

The pathological diagnosis of chronic gastritis was based on the Sydney classification and considered as controls. EGC was confined to the mucosa or submucosa as determined by surgery or endoscopic submucosal dissection (ESD) after biopsy. This study consisted of a discovery phase and a validation phase with 77 and 75 tissue samples, respectively. In the discovery phase, gene expression profiling was performed on 19 LGIN, 20 HGIN, 19 EGC, and 19 chronic gastritis tissue samples using microarrays. In the validation phase, independent tissue samples from 26 LGIN, 15 HGIN, 14 EGC, and 20 chronic gastritis patients were used in a real-time TaqMan® PCR assay (Applied Biosystems, CA, Unites States). The clinicopathological characteristics of the patients in the different groups were evaluated in terms of gender and age. The inclusion criteria were: voluntary participation in the study with informed consent and a definite pathological diagnosis by 2 pathologists. The pathologists reviewed all cases from the 2 different hospitals according to the same criteria and agreed with all the diagnosis. This study was approved by the Ethics Committee of PUMCH and also received institutional approval; the experiments were carried out in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research[8].

Formalin-fixed tissue samples

Formalin-fixed paraffin-embedded blocks of 155 specimens were obtained from patients who underwent ESD in the Departments of Gastroenterology or underwent gastrectomy in the Department of General Surgery at PUMCH between September 2010 and September 2013. Patient age ranged from 39 to 78 years with a mean of 56 years, and the male-to-female ratio was 1.47. The pathological diagnosis of 61 chronic gastritis was based on the Sydney classification. A total of 94 neoplasia were diagnosed by hematoxylin and eosin staining according to the WHO Classification of Tumors of the Digestive System, with 24 specimens classified as LGIN, 40 as HGIN, and 30 as EGC.

RNA preparation

The samples were stored in RNAlater® Solution immediately after biopsy during upper endoscopy. The samples were incubated in RNAlater® Solution overnight at 4 ℃C and then transferred to -80 ℃. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, MD, Unites States). The concentration was measured by ND-1000 UV-VIS spectrophotometry (NanoDrop Technologies, DE, Unites States). The quality of the purified RNA (RNA integrity number, RIN) was determined using the RNA 6000 LabChip Kit and Agilent 2100 Bioanalyzer (Agilent, CA, Unites States). RNA samples with an $A_{260/280}$ ratio greater than 1.8 and an RIN greater than 6.5 were included.

Gene expression microarray analysis

Sample labeling, hybridization, washing, and scanning steps were performed at the Cancer Institute and Chinese Academy of Medical Science according to the manufacturer's instructions. Initially, 100 ng total RNA was added to the reaction to generate 1.65 μg Cy3-labeled cRNA using the Low Input Quick Amp Labeling Kit (Agilent). Then, the cRNA was hybridized to an Agilent 4 × 44 K Whole Human Genome microarray. After hybridization, the slides were washed and then scanned with the Agilent G2505B Microarray Scanner System. The fluorescent intensities of the scanned images were extracted and preprocessed with the Agilent Feature Extraction Software (version 9.1). The microarray data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (GSE55696). Data normalization, quality control, principal component analysis, and filter by flags were performed using GeneSpring software GX 12.6 (Agilent Technologies), and only the detected flags were acceptable.

Real-time quantitative PCR

First, 500 ng total RNA was reverse transcribed using SuperScript™ IIReverse Transcriptase, and cDNA was produced. The gene expression was analyzed on the MX3005P ™ QPCR System (Stratagene) using TaqMan® Gene Expression Assay probes for G_0S_2 (Hs00274783_s1) and POLR2A (Hs00172187_m1) according to the manufacturer's protocol (Applied Biosystems, CA, Unites States). The PCR program was initiated at 50 ℃ for 2 min and at 95 ℃ for 10 min before 45 thermal cycles, each at 95 ℃ for 15 s and at 60 ℃ for 1 min. The gene expression levels of G_0S_2 were assessed relative to those of POLR2A. The equation $-^{\Delta CT}G_0S_2 = -(^{CT}G_0S_2 - {}^{CT}\text{POLR2A})$ represents the expression level of G_0S_2, *i.e.*, a greater $-^{\Delta CT}G_0S_2$ value indicates a higher level of G_0S_2 expression.

IHC staining

IHC analysis included a polyclonal antibody for G_0S_2 (Novus, CO, Unites States; dilution 1:100). Briefly, paraffin blocks were cut into 3-μm thickness sections. These

Table 1 Clinical characteristics of all patients

	Discovery phase (n = 77)	Validation phase (n = 75)	P value
Gender			0.48
Male	46	49	
Female	31	26	
Age (yr)			0.14
Mean ± SE (range)	58.87 ± 9.13 (36-78)	56.61 ± 9.39 (36-79)	

sections were deparaffinized in xylene and rehydrated in a descending ethanol-to-water gradient series. Endogenous peroxidase was blocked by exposure to 3% H_2O_2 for 10 min. For antigen retrieval, sections were subjected to microwave heat in citrate buffer (pH 9.0) with ebullition for 5 min. After cooling to room temperature, sections were incubated with a polyclonal antibody for G_0S_2 at room temperature for 2 h. Anti-rabbit immunoglobulin G labeled with biotin (OriGene Technologies, Beijing, China) was used as a secondary antibody for the detection of primary antibodies and was incubated at room temperature for 30 min. The tissue sections were ready for chromogen reaction with 0.02% diaminobenzidine, and then were counterstained with hematoxylin. All samples were processed under the same conditions. Human seminiferous duct cells in testis tissue served as positive control following the manufacturer's protocol.

All immunostained slides were evaluated independently by 2 pathologists (Zhou WX and Li Y). The percentage of G_0S_2 positive cells was calculated and scored as follows: score 0 = undetectable staining and from 1% to 29% of positive cells; 1 = from 30% to 59% of positive cells; 2 = from 60% to 89% of positive cells; 3 = ⩾ 89% of positive cells. The intensity of cytoplasmic staining was also evaluated for G_0S_2 and scored from 1 to 3 (from low, through medium to high). For statistical analysis, multiplying the percentage and intensity scores provided a composite expression score (0-9). A composite score of 1-4 was classified as negative and 5-9 was ranked as positive.

Statistical analysis

The statistical analyses of the gene expression microarray data were performed using an unpaired t-test and corrected with the Benjamini and Hochberg false discovery rate (FDR) algorithm. The statistical significance was assessed at $P < 0.05$ to identify genes that were differentially expressed between different types of lesions and to perform a gene ontology (GO) enrichment analysis using the GeneSpring software GX 12.6. Hierarchical clustering was performed using R (http://www.r-project.org/). SPSS (version. 18.0) software (SPSS Inc., Chicago, IL, Unites States) was used for statistical analysis of the clinical features, the qPCR results, and the IHC results. The χ^2 test was used to compare the categorical variables. Regarding the numerical variables, comparisons of parameters between 2 groups were made using an unpaired t-test, and those among 4 groups were performed using one-way analysis of variance followed by least significant difference. $P < 0.05$ was considered statistically significant.

Table 2 Clinicopathological characteristics of early-stage gastric carcinoma

	Discovery phase (n = 19)	Validation phase (n = 14)	P value
Depth of invasion			0.31
Tunica mucosa	6	2	
Muscularis mucosae	8	5	
Submucosa	5	7	
Differentiation			0.56
Well	5	4	
Moderate	4	5	
Poor	10	5	

RESULTS

Clinical characteristics of enrolled patients

In total, 152 tissue samples were randomly divided into 2 sets, with no significant differences with respect to age (P = 0.14), gender (P = 0.48), or other clinicopathological features; the sets were used in the discovery and validation phases. The clinical characteristics of all patients are summarized in Table 1. The mean age of the patients was 57.76 ± 9.30 years. Regarding the EGC group, the 2 sets showed no significant differences in terms of depth of invasion (P = 0.31) or differentiation (P = 0.56) (Table 2).

HGIN and LGIN are biologically distinct

The whole genomic expression profiles of 19 LGIN and 20 HGIN were analyzed. The transcripts whose expression level differed significantly between LGIN and HGIN were explored. In total, 2521 significantly differentially expressed transcripts (951 upregulated and 1570 downregulated in HGIN) were identified and may be involved in the development of HGIN.

To identify the functionally relevant gene clusters, a GO enrichment analysis of the 951 and 1570 above-mentioned transcripts was performed. There were no enriched GO terms among the 1570 downregulated transcripts. The enriched GO terms in the 951 upregulated transcripts were associated with metabolism, the defense response, and the NF-κB cascade, which are shown in Table 3. It was hypothesized that the upregulated transcripts contributed the most relevant functions and processes. The biological distinctions between HGIN and LGIN were primarily involved in metabolism, the defense response, and the NF-κB cascade (Table 3).

Molecular differences between HGIN and EGC are not significant

Gene expression analyses were performed in 20 HGIN and 19 EGC to reveal the molecular differences between these tissues. Unlike the marked differences between HGIN and LGIN, there were only 58 differentially expressed transcripts between HGIN and EGC, including 38 upregulated and 20 downregulated transcripts in EGC. No GO term was enriched based on the 20 downregu-

Table 3 Enriched Gene Ontology terms of the upregulated transcripts in high-grade intraepithelial neoplasia compared with low-grade intraepithelial neoplasia

GO accession	GO term	*P* value	Genes involved
GO:0043170|GO:0043283	Macromolecule metabolic process	4.01×10^{-9}	272
GO:0044260|GO:0034960	Cellular macromolecule metabolic process	3.84×10^{-9}	247
GO:0006952|GO:0002217|GO:0042829	Defense response	9.44×10^{-8}	63
GO:0005515|GO:0045308	Protein binding	1.09×10^{-7}	358
GO:0044422	Organelle part	3.35×10^{-7}	292
GO:0044428	Nuclear part	4.51×10^{-7}	145
GO:0044446	Intracellular organelle part	4.17×10^{-7}	288
GO:0070013	Intracellular organelle lumen	5.50×10^{-6}	144
GO:0043122	Regulation of I-κB kinase/NF-κB cascade	6.79×10^{-6}	21
GO:0043123	Positive regulation of I-κB kinase/NF-κB cascade	9.53×10^{-6}	18

NF-κB: Nuclear factor-κB; GO: Gene ontology.

Table 4 Enriched Gene Ontology categories of the upregulated transcripts in early-stage gastric carcinoma compared with high-grade intraepithelial neoplasia

GO accession	GO term	*P* value	Genes involved
GO:0050776	Regulation of the immune response	2.27×10^{-8}	9
GO:0050851	Antigen receptor-mediated signaling pathway	4.24×10^{-7}	5
GO:0002429	Immune response-activating cell surface receptor signaling pathway	5.82×10^{-7}	5
GO:0050863	Regulation of T cell activation	6.90×10^{-7}	6
GO:0002768	Immune response-regulating cell surface receptor signaling pathway	7.10×10^{-7}	5
GO:0050778	Positive regulation of the immune response	3.23×10^{-7}	7
GO:0002684	Positive regulation of the immune system process	3.92×10^{-7}	8
GO:0001772	Immunological synapse	1.95×10^{-6}	3
GO:0051249	Regulation of lymphocyte activation	2.53×10^{-6}	6
GO:0042102	Positive regulation of T cell proliferation	2.81×10^{-6}	4

GO: Gene ontology.

lated transcripts. Although the expression differences between HGIN and EGC were not great, it is worth noting that the GO enrichment analysis result of 38 upregulated transcripts was relatively prominent in the progression from HGIN to EGC. The molecular differences between HGIN and EGC mainly focused on the immune response, as shown in Table 4.

Comparison with LGIN indicates that some of the alterations associated with HGIN are retained in EGC

When compared with LGIN, among the 951 upregulated genes in HGIN, 289 transcripts (Figure 1) were expressed at a similarly high level in EGC and were involved in the GO enrichment analysis. Indeed, the 289 transcripts could separate both HGIN and EGC from the LGIN class. The heat map in Figure 2 shows their clear separation. These transcripts that were overexpressed in HGIN compared with LGIN were also overexpressed at similar or even higher levels in EGC. In terms of GO enrichment, some of the alterations associated with HGIN compared with LGIN, such as metabolism, the defense response, and the NF-κB cascade, were still retained in EGC.

Significant variation of G_0S_2 in HGIN and early-stage adenocarcinoma vs LGIN within a microarray analysis

G_0S_2 was related to metabolism, the defense response, and the NF-κB cascade and was overexpressed in both HGIN and EGC compared with LGIN.

As shown in Table 5, the expression level of G_0S_2 in gastric EGC tissue was 6.00-fold higher than in gastric LGIN tissue (unpaired *t*-test with FDR correction *P*corr < 0.001) (Table 5). The expression level of G_0S_2 in gastric HGIN tissue was 3.28-fold higher than in gastric LGIN tissue (*P*corr < 0.05). Between HGIN and early-stage adenocarcinoma, the expression differences of G_0S_2 were not statistically significant (*P*corr > 0.05). However, the expression of EGC was 1.83 fold higher than that of HGIN (Figure 3).

Compared with gastritis, the overexpression of G_0S_2 in HGIN and EGC was statistically significant (*P*corr < 0.01). The difference between LGIN and gastritis was not statistically significant. The expression of G_0S_2 was relatively higher in the malignant lesions than in the benign lesions (Figure 3).

Independent validation in quantitative real-time PCR analysis

To validate the above results, qPCR of G_0S_2 was performed to quantify the RNA levels in independent tissue samples. The expression of G_0S_2 was elevated in HGIN and early-stage adenocarcinoma compared with LGIN, which was identical to the microarray results (Figure 4). Compared with LGIN, the difference in G_0S_2 expression

Table 5 Statistical results of the differential expression of G_0S_2 among tissue samples in the microarray data

Unpaired *t*-test of G0S2	*P* value (corr)	*P* value	FC (abs)	Direction	Probe name	Gene symbol description
HGIN *vs* LGIN	0.021	0.001	3.28	Up in HGIN	A_23_P74609	Homo sapiens G_0/G_1 switch 2 (G_0S_2), mRNA NM_015714
EGC *vs* LGIN	< 0.001	< 0.001	6.00	Up in EGC		
HGIN *vs* Gastritis	0.005	0.001	3.19	Up in HGIN		
EGC *vs* Gastritis	< 0.001	< 0.001	5.83	Up in EGC		
EGC *vs* HGIN	0.463	0.103	1.83	Up in EGC		
LGIN *vs* Gastritis	0.946	0.927	1.03	Down in LGIN		

HGIN: High-grade intraepithelial neoplasia; EGC: Early-stage adenocarcinoma; LGIN: Low-grade intraepithelial neoplasia.

Table 6 Statistical results of the differential expression of G_0S_2 among tissue samples in immunohistochemical staining data *n* (%)

	G_0S_2 < 5 (*n* = 131)	G_0S_2 ≥ 5 (*n* = 24)	Pearson χ^2 of 4 groups
Gastritis (*n* = 61)[1-3]	61 (100.0)	0 (0)	*P* < 0.001
LGIN (*n* = 24)	21 (87.5)	3 (12.5)	
HGIN (*n* = 40)[4,5]	23 (57.5)	17 (42.5)	
EGC (*n* = 30)	26 (86.7)	4 (13.3)	

[1]Compared with low-grade intraepithelial neoplasia (LGIN), *P* = 0.005; [2]Compared with HGIN, *P* < 0.001; [3]Compared with EGC, *P* = 0.004; [4]Compared with LGIN, *P* = 0.012; [5]Compared with EGC, *P* = 0.008. HGIN: High-grade intraepithelial neoplasia; EGC: Early-stage adenocarcinoma.

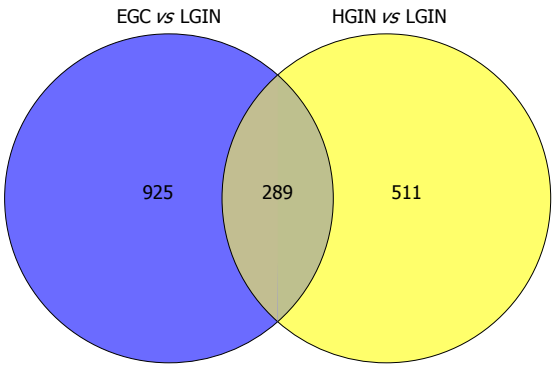

Figure 1 All of the transcripts that were statistically significant both in gastric early-stage adenocarcinoma *vs* low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia *vs* low-grade intraepithelial neoplasia and were involved in the Gene Ontology enrichment analysis. LGIN: Low-grade intraepithelial neoplasia; EGC: Early-stage adenocarcinoma; HGIN: High-grade intraepithelial neoplasia.

was significant both in EGC and HGIN ($P < 0.001$ and $P < 0.01$, respectively) (Figure 4). Similarly, compared with gastritis, the difference in G_0S_2 expression was significant in EGC and HGIN ($P < 0.001$ and $P < 0.05$, respectively).

Validation in IHC analysis

G_0S_2 immunoreactivity was detected in the cytoplasmic of neoplastic cells, but was undetectable (Figure 5A) in chronic gastritis cells (gastritis *vs* LGIN, $P = 0.005$; gastritis *vs* HGIN, $P < 0.001$; gastritis *vs* EGC, $P = 0.004$). The expression rates of G_0S_2 were 12.5% in LGIN (Figure 5B), 42.5% in HGIN (Figure 5C), and 13.3% in EGC (Figure 5D). The G_0S_2 expression in HGIN was higher than that of LGIN ($P = 0.012$, $\chi^2 = 6.28$) and EGC ($P = 0.008$, $\chi^2 = 6.94$) (Table 6).

In conclusion, HGIN and LGIN are biologically distinct, and the expression of G_0S_2 is significantly elevated in HGIN and EGC compared with LGIN. As a NF-κB cascade-and metabolism-related gene, G_0S_2 may be of considerable interest in the malignant transformation of gastric intraepithelial neoplasia.

DISCUSSION

According to the Vienna classification of GIN, patients with HGIN or carcinoma (CIS, suspicious, intramucosal, or submucosal invasion) should undergo resection (endoscopic or surgical). For patients with LGIN, endoscopic resection or follow-up is indicated[9]. There is evidence that genetic factors are related to gastric cancer and relevant precancerous lesions[10]. Gastric precancerous lesions are currently distinguished into LGIN and HGIN. Several large-scale follow-up studies have revealed that patients with HGIN are at higher risk of developing gastric carcinoma than are patients with LGIN. In this study, the gene expression profiles of gastric LGIN, HGIN, and EGC tissue were analyzed. There were 951 significantly upregulated transcripts and 1570 significantly downregulated transcripts in HGIN compared with LGIN. However, there were no enriched GO terms among the 1570 downregulated transcripts when a GO enrichment analysis was performed. Similarly, there were 38 upregulated and 20 downregulated transcripts in EGC compared with HGIN and no GO term was enriched based on the 20 downregulated transcripts. It was supposed that the transcripts contributed most to gastric early carcinogenesis were upregulated along with the progression from LGIN, through HGIN to EGC. The gene expression patterns between HGIN and LGIN were distinct, and the enriched GO terms in the above-mentioned 951 differentially expressed transcripts were associated with metabolism, the defense response, and the NF-κB cascade. Similarly, the differences in gene expression profiling between EGC and LGIN were significant with the parallel enriched GO terms. Presumably, the biological similarities between HGIN and EGC were prominent when compared with LGIN. However, the molecular differences between HGIN and EGC were not significant and the involved function of their differentially expressed transcripts were mainly focused on the immune response.

The genes that are involved in metabolism, the de-

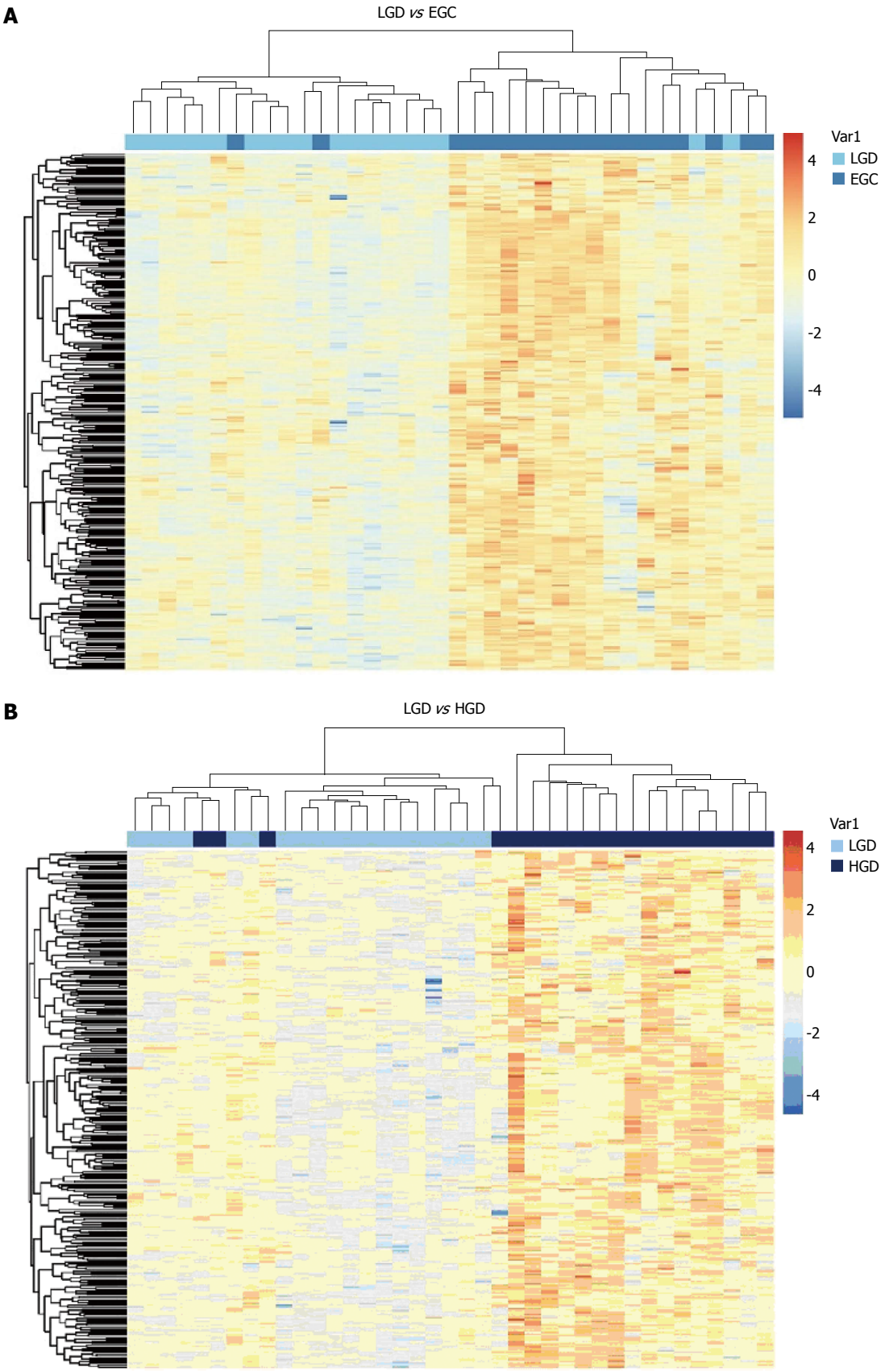

Figure 2 Unsupervised cluster analysis of gastric low-grade intraepithelial neoplasia and early-stage adenocarcinoma (A), low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia (B) tissue samples with the 289 differentially expressed transcripts. The transverse blue bar indicates the histopathological type of the tissue samples: A: Light blue, low-grade intraepithelial neoplasia (LGIN); Dark blue, early-stage adenocarcinoma (EGC); B: Light blue, LGIN; Dark blue, high-grade intraepithelial neoplasia (HGIN). Each column represents a sample, and each row represents a transcript. The expression abundance from low levels to high levels is shown in colors from blue to red.

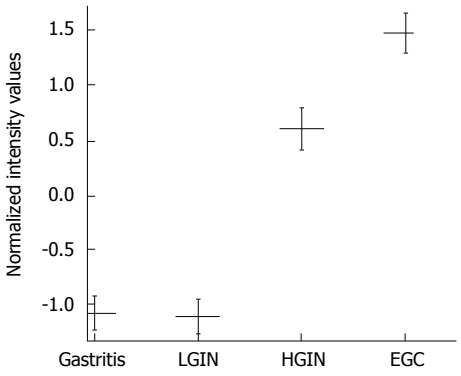

Figure 3 G_0S_2 expression is increased in gastric high-grade intraepithelial neoplasia and early-stage adenocarcinoma in the microarray. The G_0S_2 mRNA expression in gastric high-grade intraepithelial neoplasia (HGIN), tissue shows a statistically significant elevation compared with that of low-grade intraepithelial neoplasia (LGIN). No statistically significant difference in G_0S_2 expression was found between HGIN and early-stage adenocarcinoma (EGC). The results are expressed as the mean ± SE.

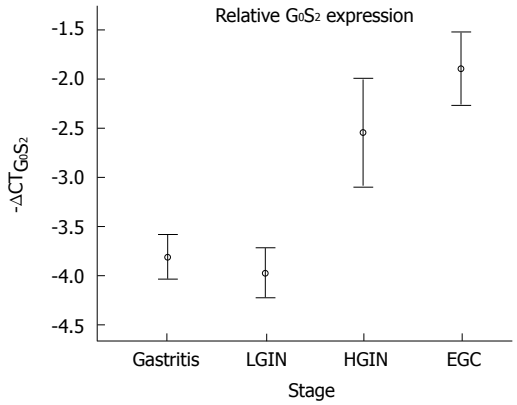

Figure 4 G_0S_2 expression was validated by quantitative polymerase chain reaction in the independent samples. The results are shown as the mean ± SE. -ΔCTG_0S_2 represents the expression level of G_0S_2. The differences of -ΔCTG_0S_2 in the 4 groups [low-grade intraepithelial neoplasia (LGIN), high-grade intraepithelial neoplasia (HGIN), early-stage adenocarcinoma (EGC) and gastritis] were compared using an analysis of variance (one-way ANOVA). A test for the homogeneity of variances shows $P = 0.105$, indicating that the variances are equal. The mean difference between LGIN and HGIN was statistically significant ($P = 0.004$). The mean difference between LGIN and EGC was statistically significant ($P < 0.001$).

fense response, and the NF-κB cascade were not only upregulated in HGIN compared with LGIN but were also upregulated at similar or even higher expression levels in EGC. A characteristic gene related to metabolism, the immune response, and the NF-κB cascade, G_0S_2, was subsequently validated in independent samples using a qPCR test and an IHC test. Although the microarray analysis and qPCR analysis showed that G_0S_2 expression was higher in EGC and HGIN than in LGIN, IHC staining results showed the expression rate of G_0S_2 was higher in HGIN but similar in LGIN and EGC. The expression level of mRNA and IHC staining may be not the same.

G_0S_2 is located on chromosome 1 of the genome and encodes a small basic protein of 103 amino acids. G_0S_2 is related to the re-entry of cells from the G_0 phase to the G_1 phase of the cell cycle, implying that the G_0S_2 gene has a cell proliferative function. Previous studies have indicated that G_0S_2 is highly induced through NF-κB following tumor necrosis factor-α treatment[11]. G_0S_2 is a novel target gene of PPARs (peroxisome proliferator-activated receptors, α, β/β, and γ), which are mainly involved in lipid metabolism[12,13]. Activation of PPARγ inhibits cell growth and induces apoptosis in gastric cancer cells. G_0S_2 is an inhibitor of adipose triglyceride lipase and decreases lipolysis[14-17]. G_0S_2 is a positive regulator of oxidative phosphorylation[18]. G_0S_2 was upregulated in inflammatory processes, was involved in T cell quiescence, and inhibited proliferation of T cells. Inhibition of MAPK, PI3K, mTOR and Ca^{2+}/calcineurin pathways abolished G_0S_2 gene suppression[19-21].

NF-κB transcription factor and its signaling pathway play a key role in controlling the immune response[22-24]. NF-κB provides a mechanistic link between inflammation and cancer[25-27]. NF-κB controls the ability of preneoplastic and malignant cells to resist apoptosis-based tumor-surveillance mechanisms, which is crucial for the clinical development of NF-κB inhibitors for cancer therapy[28]. The alterations in certain aspects of lipid metabolism were involved in tumorigenesis, including the synthesis and degradation of lipids that contribute to energy homeostasis and the abundance of lipids with signaling functions[29-32].

Previous studies with HGIN and LGIN were mostly based on several known genes[33,34], such as *p53*, *Her-2*, and E-cadherin[35]. Until now, few studies have focused on GIN, especially from the aspect of the whole genome expression profile.

The microarray approach has been widely applied to the investigation of advanced gastric cancer. However, advanced gastric cancer has a poor prognosis and greater heterogeneity with its progression. Several studies have revealed that genetic alterations begin in the early stages of cancer and even in precancerous lesions. Early studies using CGH arrays suggested that an 8q gain in HGIN may play a pivotal role in the development of gastric carcinoma[7]. However, the gene expression levels and functions associated with the copy number status of 8q were not detailed. Much less is known in terms of a comparison among the expression profiles of LGIN, HGIN, and EGC due to the difficulties of early detection and biopsy. Based on the clinicopathological findings and a clear research design, this study collected samples of LGIN, HGIN, and EGC to perform a comprehensive determination of their expression profiles at a whole-genome level. Through this study of gene expression profiles, the biological basis of the clinical differences between HGIN and LGIN was determined.

Despite the limitation that a larger number of samples in each group would have been beneficial, this large-scale study opens the door for further research into precancerous lesions. The asymptomatic characteristics, atypical histology, and lack of specific biomarkers in precancerous lesions[10,36] make them difficult to diagnose early and accurately. Moreover, the performance of the gene in IHC

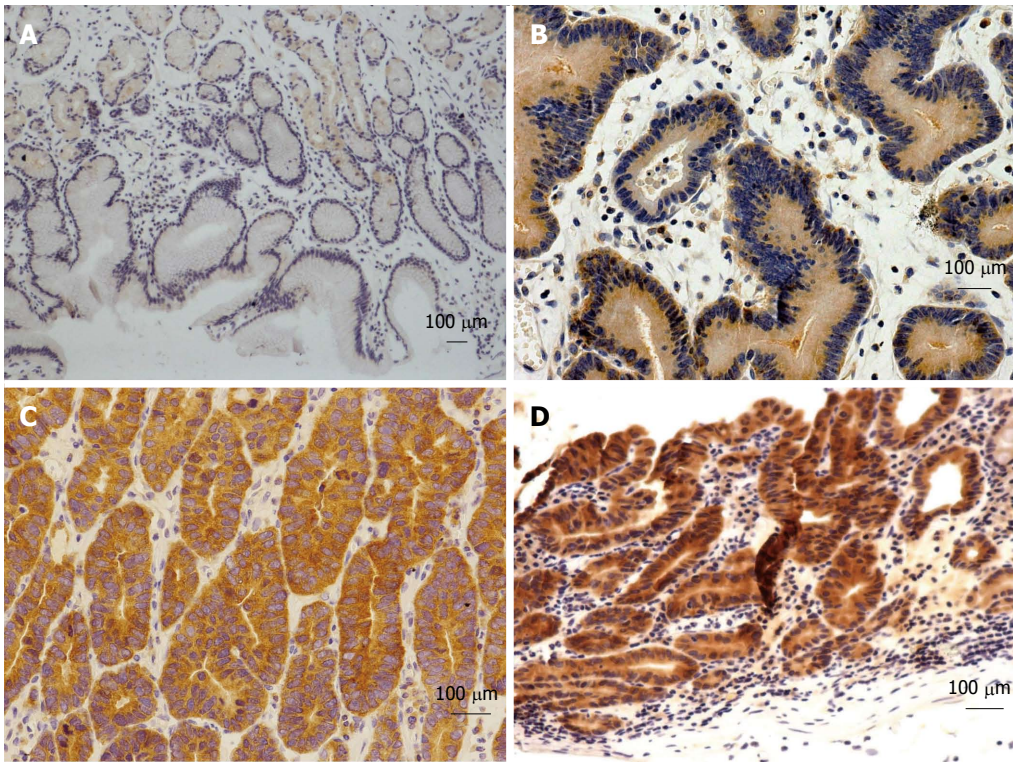

Figure 5 Representative immunohistochemistry for G_0S_2 in gastric samples. Representative samples are shown from G_0S_2 (A) chronic gastritis tissues with negative expression, and G_0S_2 (B) low-grade intraepithelial neoplasia tissues with low expression, G_0S_2 (C) high-grade intraepithelial neoplasia tissues with medium expression, and G_0S_2 (D) early-stage adenocarcinoma tissues with high expression (bar represents 100 μm).

experiments may be useful for early detection of GIN, and clinical application for auxiliary diagnosis of atypical lesions is promising. Furthermore, the gene function-related research requires future study.

In summary, the microarray analysis in this study identified differences in the gene expression between LGIN and HGIN. The obvious molecular distinction between LGIN and HGIN was almost identical in different clinical practices. Metabolism, the immune response, and the NF-κB cascade appear to be important processes underlying malignant transformation.

ACKNOWLEDGMENTS

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COMMENTS

Background

Early detection and an appropriate diagnosis of gastric intraepithelial neoplasia (GIN) and early-stage cancer are associated with improved outcomes for patients. It is important to understand more about these stages both clinically and biologically. However, little is known about their biological characteristics due to the difficulties of early detection and biopsy.

Research frontiers

Low-grade GIN (LGIN) and high-grade GIN (HGIN) apparently have different clinicopathological characteristics. Previous gene expression profiling studies on gastric precancerous lesions did not detail the differences between LGIN and HGIN. Based on the potential transition between and morphological similarity of dysplasia and carcinoma, the hypothesis that they are biologically related is reasonable.

Innovations and breakthroughs

This is the first study to perform a comprehensive detection of the gene expression profiling of LGIN and HGIN and early-stage adenocarcinoma. The characteristic upregulated genes during gastric early carcinogenesis were involved in metabolism and the immune response and nuclear factor-κB (NF-κB) pathway, and their expression was validated in independent samples through real-time TaqMan® PCR and immunohistochemical staining.

Applications

This study collected specific samples to report the clear distinction of the gene expression profiles between LGIN and HGIN, thus providing molecular evidence for their different clinical relevance. G_0S_2 was identified as a potential marker to differentiate LGIN and HGIN.

Terminology

NF-κB transcription factor and its signaling pathway play a key role in controlling the immune response. NF-κB provides a mechanistic link between inflammation and cancer. NF-κB controls the ability of pre-neoplastic and malignant cells to resist apoptosis-based tumor-surveillance mechanisms, which is crucial for the clinical development of NF-κB inhibitors in cancer therapy. G_0S_2 is related to the re-entry of cells from the G_0 phase to the G_1 phase of the cell cycle. G_0S_2 is an inhibitor of adipose triglyceride lipase and decreases lipolysis, and is a positive regulator of oxidative phosphorylation.

Peer review

This manuscript presents an interesting differential gene expression profiling of gastric LGIN, HGIN and early gastric cancer, and try to explore the molecular alteration in the malignant progression of gastric neoplasia. From their microarray data, authors identified G_0S_2, a putative lymphocyte G_0/G_1 switch gene product as a potential marker to differentiate low grade neoplastic lesion from high grade lesion in the stomach.

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ORIGINAL ARTICLE

HIF-1α induces VE-cadherin expression and modulates vasculogenic mimicry in esophageal carcinoma cells

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Author contributions: Tang NN, Wang L and Jin HL performed the majority of experiments; Zhang WF, Hao B and Wang P provided vital reagents and analytical tools; He GJ collected all the human materials; Shi RH designed the study; Tang NN wrote the manuscript; Zhang HJ and Zhu H revised the manuscript.

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Abstract

AIM: To investigate whether hypoxia inducible factor (HIF)-1α modulates vasculogenic mimicry (VM) by upregulating VE-cadherin expression in esophageal squamous cell carcinoma (ESCC).

METHODS: Esophageal squamous cancer cell lines Eca109 and TE13 were transfected with plasmids harboring small interfering RNAs targeting HIF-1α or VE-cadherin. The proliferation and invasion of esophageal carcinoma cells were detected by MTT and Transwell migration assays. The formation of tubular networks of cells was analyzed by 3D culture *in vitro*. BALB/c nude mice were used to observe xenograft tumor formation. The relationship between the expression of HIF-1α and VE-cadherin, ephrinA2 (EphA2) and laminin5γ2 (LN5γ2) was measured by Western blot and real-time polymerase chain reaction.

RESULTS: Knockdown of HIF-1α inhibited cell proliferation (32.3% ± 6.1% for Eca109 cells and 38.6% ± 6.8% for TE13 cells, $P < 0.05$). Both Eca109 and TE13 cells formed typical tubular networks. The number of tubular networks markedly decreased when HIF-1α or VE-cadherin was knocked down. Expression of VE-cadherin, EphA2 and LN5γ2 was dramatically inhibited, but the expression of matrix metalloproteinase 2 had no obvious change in HIF-1α-silenced cells. Knockdown of VE-cadherin significantly decreased expression of both EphA2 and LN5γ2 ($P < 0.05$), while HIF-1α expression was unchanged. The time for xenograft tumor formation was 6 ± 1.2 d for Eca109 cells and Eca109 cells transfected with HIF-1α Neo control short hairpin RNA (shRNA) vector, and 8.4 ± 2.1 d for Eca109 cells transfected with an shRNA against HIF-1α. Knockdown of HIF-1α inhibited vasculogenic mimicry (VM) and tumorigenicity *in vivo*.

CONCLUSION: HIF-1α may modulate VM in ESCC by regulating VE-cadherin expression, which affects VM formation through EphA2 and LN5γ2.

Key words: Esophageal squamous cell carcinoma; Hypoxia-inducible factor-1α; VE-cadherin; RNA interference; Vasculogenic mimicry

Core tip: Hypoxia-inducible factor (HIF) is a key factor in regulating and promoting tumor progression. Angiogenesis and vasculogenic mimicry (VM) may play an important role in tumor acquisition of increased blood supply. We investigated the role of HIF-1α in the formation of VM in esophageal squamous cell carcinoma (ESCC). We showed that HIF-1α may upregulate the expression of VE-cadherin to modulate VM in ESCC, which may be

related to changes in ephrin A2 and laminin 5γ2 protein expression. These results may have implications for the treatment of malignant tumor diseases.

Tang NN, Zhu H, Zhang HJ, Zhang WF, Jin HL, Wang L, Wang P, He GJ, Hao B, Shi RH. HIF-1α induces VE-cadherin expression and modulates vasculogenic mimicry in esophageal carcinoma cells. *World J Gastroenterol* 2014; 20(47): 17894-17904 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17894.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17894

INTRODUCTION

As tumors grow, their microenvironment becomes increasingly hypoxic. Under hypoxic conditions, a signaling pathway involving a crucial oxygen response regulator, termed hypoxia-inducible factor (HIF), is switched on. The α subunit of HIF-1 (HIF-1α) is a nuclear factor that is generally present in mammals, and is a well-established mediator in cancer response to hypoxia[1]. HIF-1α is degraded shortly after expression in the cytoplasm under normoxic conditions. However, HIF-1α protein can be translocated into the nucleus where it is combined with the β subunit of HIF-1 to form the HIF-1 heterodimer under hypoxic conditions. Research has shown that HIF-1α is related to angiogenesis and vasculogenic mimicry (VM)[2,3].

As we all know, angiogenesis is not the only mechanism by which tumors acquire a blood supply. Highly aggressive and metastatic melanoma cells can form vascular channel-like structures that are independent of angiogenesis. This phenomenon is called VM[3]. Tumor cell VM describes the functional plasticity of aggressive cancer cells forming *de novo* vascular networks. The initial morphological and molecular characterization of VM in human melanoma showed that the tumor cells coexpressed endothelial and tumor markers and formed channels, networks, and tubular structures. This provides a perfusion pathway for rapidly growing tumors, transporting fluid from leaky vessels, and/or connecting with endothelial-lined vasculature as well as an escape route for metastasis.

Recent research has suggested that tumors can be viewed as highly heterogeneous populations derived from one common progenitor. As suggested by Grunewald *et al*[4], although the degree to which cancer cells resemble endothelial cells is debatable, cancer cells can directly line the lumen of functional tumor blood vessels. Moreover, like the foragers in ant colonies, these cancer cells do not reproduce, but instead enable tumor growth indirectly by attraction of heterotypic tissues through chemotactic substances [*e.g.*, vascular endothelial growth factor (VEGF)], in the same way that ants attract and recruit nestmates and even prey by odor trails and pheromones. Since the introduction of VM, many studies have contributed mechanistic insights into VM in a variety of cancers. In particular, critical VM-modulating genes are associated with vascular [VE-cadherin, ephrinA2 (EphA2) and VEGF] and hypoxia-related (HIF and Twist1) signaling pathways.

HIF-1α-siRNA significantly suppressed the VM networks under either normoxic or hypoxic conditions in gallbladder carcinoma[5]. Su *et al*[6] have suggested that a hypoxic microenvironment increases HIF-1α expression and induces the formation of VM channels to acquire an adequate blood supply in ovarian cancer cells.

VE-cadherin is a master gene for both tumor angiogenesis and VM[7-9]. Overexpression of VE-cadherin in various vasculogenic tumor cells has been implicated in tumor neovascularization, growth, and progression[10]. Accordingly, VE-cadherin is proposed as a target for antiangiogenic drug discovery and anti-cancer therapy[11]. HIF-1 is combined with the core recognition sequence 5'-RCGTG-3' of the promoter sequence of hypoxia-inducible genes to promote transcription and translation of these genes[12]. There is the 5'-ACGTG-3' sequence in the promote region of *VE-cadherin* gene. Therefore, we speculate that *VE-cadherin* may be one target gene of HIF-1α, which plays an important role in the development of VM in esophageal squamous cell carcinoma (ESCC).

This study was designed to observe the formation of vascular-network-like structures in ESCC cell lines and the impact of HIF-1α and VE-cadherin on VM in ESCC. Furthermore, the possible molecular mechanism by which HIF-1α modulates VM in ESCC cells was investigated.

MATERIALS AND METHODS

Cell culture

ESCC cell lines Eca109 and TE13 were obtained from Cell Resource Center of Shanghai Life Science Institute. In former work, we established Eca109 and TE13 cells stably transfected with an short hairpin (sh)RNA targeting HIF-1α, which were designated as Eca109/HIF-1α shRNA cells and TE13/HIF-1α shRNA cells, respectively. The protein gel blot results demonstrated that compared to untransfected cells or cells transfected with HIF-1α Neo control shRNA vector, HIF-1α level was significantly decreased in shRNA-transfected cells[13]. Eca109 and TE13 cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT, United States) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone) at 37 ℃ in a humidified atmosphere containing 5% CO_2/95% air, or hypoxic treatment was given by placing cells in a hypoxia chamber flushed with a gas mixture comprising 1% O_2/5% CO_2/94% N_2. Eca109/HIF-1α shRNA and TE13/HIF-1α shRNA cells were cultured in the same environment.

RNA interference

A set of three shRNA constructs directed against human VE-cadherin mRNA and one negative control (Neo) were purchased from Shanghai Shengneng Gaming Biological Technology Company. Eca109 and TE13 cells were transfected with the VE-cadherin shRNA

constructs or VE-cadherin control construct using Lipofectamine 2000 reagent (Invitrogen, United States) according to the manufacturer's instructions. After transfection, 400 μg/mL G418 (Sigma, United States) was added to medium to select stable knockdown cells. The clones were characterized by real-time polymerase chain reaction (PCR) and Western blot to assess the level of silencing of VE-cadherin.

The stable cell lines in which HIF-1α was efficiently knocked down were named Eca109/shVE-cad derived from the Eca109 cell line and TE13/shVE-cad derived from the TE13 cell line, and the stable control cell lines were named Eca109/shVE-cad Neo and TE13/shVE-cad Neo.

The sequences of the three shRNA constructs against human VE-cadherin mRNA and the negative control were as follows: pGCsi-VE-cadherin 1: 5'-TGC TGA TGT CTT GCA GAG TGA CCA GCG TTT TGG CCA CTG ACT GAC GCT GGT CAC TGC AAG ACA T-3' and 5'-CCT GAT GTC TTG CAG TGA CCA GCG TCA GTC AGT GGC CAA AAC GCT GGT CAC TCT GCA AGA CAT C-3'; pGCsi-VE-cadherin 2: 5'-TGC TGT AAG ATG GCT ACC ACT GCC TGG TTT TGG CCA CTG ACT GAC CAG GCA GTT AGC CAT CTT A-3' and 5'-CCT GTA AGA TGG CTA ACT GCC TGG TCA GTC AGT GGC CAA AAC CAG GCA GTG GTA GCC ATC TTA C-3'; and pGCsi- VE-cadherin 3: 5'-TGCTG AAA TGT ACT GCG CGT GGA GAC GTT TTG GCC ACT GAC TGA CGT CTC CAC GCA GTA CAT TT-3' and 5'-CCTG AAA TGT ACT GCG TGG AGA CGT CAG TCA GTG GCC AAA ACG TCT CCA CGC GCA GTA CAT TTc-3'.

MTT assay

Cells were seeded into 96-well plates at 1×10^4 cells/well (100 μL) and cultured for 7 d. In the following days, the medium was removed and 20 μL MTT solution (500 μg/mL) was added to each well, followed by 4 h incubation. MTT solution was replaced by DMSO to dissolve blue formazan crystals and absorbance was measured at 570 nm using a microplate reader.

Flow cytometry analysis of apoptosis

Cells were seeded into 100-mm dishes and allowed to grow to 90% confluence. The cells were collected by digestion with EDTA-free trypsin (Invitrogen). The cell pellet was washed with cold phosphate-buffered saline (PBS) twice and resuspended in 250 μL Annexin V binding buffer (10 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 2.5 nmol/L $CaCl_2$, 1 mmol/L $MgCl_2$ and 4% bovine serum albumin). The cells were stained with Annexin V fluorescein isothiocyanate (FITC) for 15 min in the dark and subjected to flow cytometry analysis within 1 h.

3D cell culture

Matrigel (300 μL/hole) was added to 24-well plates on ice and then incubated at 37 ℃ for 30 min. Tumor cells (5×10^5/mL) were then seeded onto the gels and incubated at 37 ℃ in 5% CO_2/95% air. The cells were maintained in DMEM supplemented with 10% FBS. The tube-like structures in tumor cells were observed 12 h later. Five visual fields (up, down, left, right and center) were randomly chosen from each hole under an inverted microscope (Carl Zeiss, GER) to count the number of tube-like structures. The VM channels were identified by scanning at low power (magnification × 100 and magnification × 200). Ten non-overlapping fields at magnification × 400 were chosen to determine the median value of the VM channels. The number of VM channels was assessed using an ocular grid and the forbidden lines method to facilitate the counting.

Western blot

The cells were washed twice with ice-cold PBS and harvested in 100-200 μL cold lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 2% SDS, 4 mmol/L EDTA, 1 mmol/L PMSF and 10 U/mL aprotinin and 1 %(v/v) Triton X-100]. Lysates were kept on ice for 30 min and sonicated for 24 s using ultrasonic cell disrupter JY96-II (Ningbo, China). The extract was then centrifuged at 12000 rpm for 10 min at 4 ℃ and the supernatant was collected. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, United States). Protein (40 μg) was loaded in each lane and separated by 10% SDS-polyacrylamide gel electrophoresis at 40 mA for 90 min. The protein was transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Roche, CH) at 100 V for 70 min, which was blocked with 5% non-fat milk in TBS-T (100 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20) at room temperature for 1 h. Subsequently, the membrane was incubated with the indicated primary antibodies [(EphA2 1:500, HIF-1α 1:500, VE-cadherin 1:500, LN5γ2 1:200, matrix metalloproteinase (MMP)2 1:2000, β-actin 1:8000, α-tubulin 1:5000)] overnight at 4 ℃ and then incubated with the secondary antibodies for 1 h at 37 ℃. Bands were visualized using the ECL Western Blotting Detection System (Pierce) according to the manufacturer's instructions.

Animal xenograft model

Protocols used in the animal experiment have been approved by the institutional animal ethics committee. All procedures were performed on thirty 6-wk-old female BALB/c nude mice (Nanjing Medical University Animal Centre). The mice were randomly divided into three groups (n = 10 for each). For tumor formation evaluation, 4×10^6 cells/0.2 mL (Eca109, Eca109/Neo and Eca109/shHIF) were suspended and injected subcutaneously near the shoulder and back area. Mice in Group A, B and C received an injection of Eca109 cells, Eca109/Neo cells, and Eca109/shHIF cells, respectively. Tumor size was determined by caliper measurements of length and width. Tumor volume was calculated using the following formula: $(\text{length} \times \text{width})^2/2$. Four weeks after injection, the mice were sacrificed. Tumors were harvested, fixed with formalin and embedded in paraffin.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized, hydrated in a graded ethanol series, and rinsed with PBS. Antigens were retrieved by heating sections in a steam cooker for 30 min. Endogenous peroxide was inactivated with 3% H_2O_2 inhibitor in PBS for 12 min. Nonspecific binding was blocked in 5% horse serum and 1% goat serum for 20 min. Slides were incubated overnight at 4 ℃ with Gal-3 (1:1000), interleukin-8 (1:25; Biosource International, Camarillo CA, United States), or MMP-2 (1:400; Chemicon, Temecula, CA, United States) antibody, and next day with a peroxidase-labeled anti-rabbit antibody (1:500; Jackson Immunoresearch,United States) for 1 h at room temperature. Signaling was detected with 3, 3-diaminobenzidine (DAB; Phoenix Biotechnologies, San Antonio, TX, United States) substrate for 6 min, and the slides were counterstained with Gill's No. 3 hematoxylin (Sigma) for 20 s.

CD34-periodic acid Schiff dual staining

C918 xenograft specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin-embedded specimens were cut into serial 5-μm sections. The sections were deparaffinized, rehydrated, and subjected to immunohistochemical and periodic acid Schiff (PAS) double staining. Immunohistochemistry was conducted with a mouse monoclonal antibody against the endothelium marker CD34 (1:50 dilution; Zhong Shan Goldenbridge, Beijing, China) to identify the endothelium. DAB chromogen was used for immunohistochemistry. CD34 staining helped to distinguish the PAS-positive network of VM from endothelium-lined microvessels. Tissues were stained with PAS to identify the matrix-associated vascular channels of uveal melanoma. Quantification of VM was performed as follows: CD34-PAS dual stained sections were viewed at magnification × 400. The channels defined as VM were lined by PAS-positive material with red cells in the center of the channels, but not lined by CD34-positive endothelial cells. The mean VM count of 10 areas was calculated as the VM density (VMD) for each section. The mean VMD from five xenograft specimens in the genistein and control groups was obtained as the final VMD count.

Statistical analysis

Data were evaluated for statistical significance by one-way ANOVA with SPSS software version 11.0. All data are expressed as mean ± SE, accompanied by the number of experiments performed independently. $P < 0.05$ was considered statistically significant.

RESULTS

Knockdown of HIF-1α inhibits proliferation and migration of ESCC cells

MTT and Transwell migration assays demonstrated that Eca109/HIF-1α shRNA and TE13/HIF-1α shRNA cells proliferated and invaded slower than normal Eca109 and TE13 cells or Eca109 HIF-1α Neo and TE13 HIF-1α Neo cells (Figure 1). On day 7, knockdown of HIF-1α led to 32.3% ± 6.1% inhibition of Eca109 cell proliferation and 38.6% ± 6.8% inhibition of TE13 cell proliferation (Figure 1A). These results suggest that HIF-1α could promote the proliferation and invasion of esophageal carcinoma cells ($P < 0.05$).

Knockdown of HIF-1α expression inhibits VM formation and expression of VM-related genes in vitro

Eca109 and TE13 cells on Matrigel could connect with each other, and then formed a vascular network-like structure. The channel-forming abilities of Eca109/shHIF and TE13/shHIF cells *in vitro* were significantly inhibited, which demonstrated that the cyclic structure fractured and the number of tubular structures in the stable transfection group was markedly less than that in the control group (Figure 2A). The expression of HIF-1α, EphA2, VE-cadherin and laminin (LN)5γ2 proteins in TE13 and TE13/Neo (Eca109 and Eca109/Neo) cells under hypoxic conditions was increased compared with those under normoxia ($P < 0.05$), and expression of MMP2 had an increasing but nonsignificant trend ($P > 0.05$). Expression of HIF-1α, EphA2, VE-cadherin and LN5γ2 was notably inhibited in Eca109/shHIF and TE13/shHIF cells under normoxic conditions ($P < 0.05$) and expression of MMP2 did not change obviously. However, expression of HIF-1α and the four VM-related genes in TE13/shHIF and Eca109/shHIF cells under hypoxia was not increased compared with that under normoxia ($P > 0.05$) (Figure 2B).

HIF-1α knockdown inhibits tumorigenicity and VM structure in vivo

To validate the above *in vitro* findings *in vivo*, we established xenograft tumors by subcutaneous injection of HIF-1α knockdown Eca109 cells or the corresponding control cells into the flanks of BALB/c nude mice. The time for tumor formation was 6 ± 1.2 d for Eca109 and Eca109 HIF-1α Neo cells, and 8.4 ± 2.1 d for Eca109 HIF-1α shRNA cells. Four weeks after tumor formation, the size and weight of tumors derived from Eca109/shHIF-1α cells were significantly smaller (1.61 ± 0.29 cm^3 and 1.04 ± 0.16 g) than those derived from Eca109 cells (2.96 ± 0.69 and 2.02 ± 0.28 g), and Eca109/HIF-1α Neo cells (2.69 ± 0.63 cm^3 and 1.83 ± 0.39 g) (Figure 3A) ($P < 0.05$). Knockdown of HIF-1α significantly inhibited xenograft growth *in vivo*.

As shown in Figure 3B, the VM structure was found in Eca109 and Eca109/Neo shHIF cells cultured in 3D collagen gels more than in Eca109/shHIF cells. We examined the effects of HIF-1α knockdown on the expression of HIF-1α, EphA2, VE-cadherin and MMP2 *in vivo*. Protein gel blot analysis demonstrated that the expression levels of EphA2 and VE-cadherin were significantly lower in tumor tissues derived from Eca109/shHIF-1α cells than in those derived from Eca109 and Eca109 HIF-1α Neo control cells (Figure 3C). However, MMP2 protein

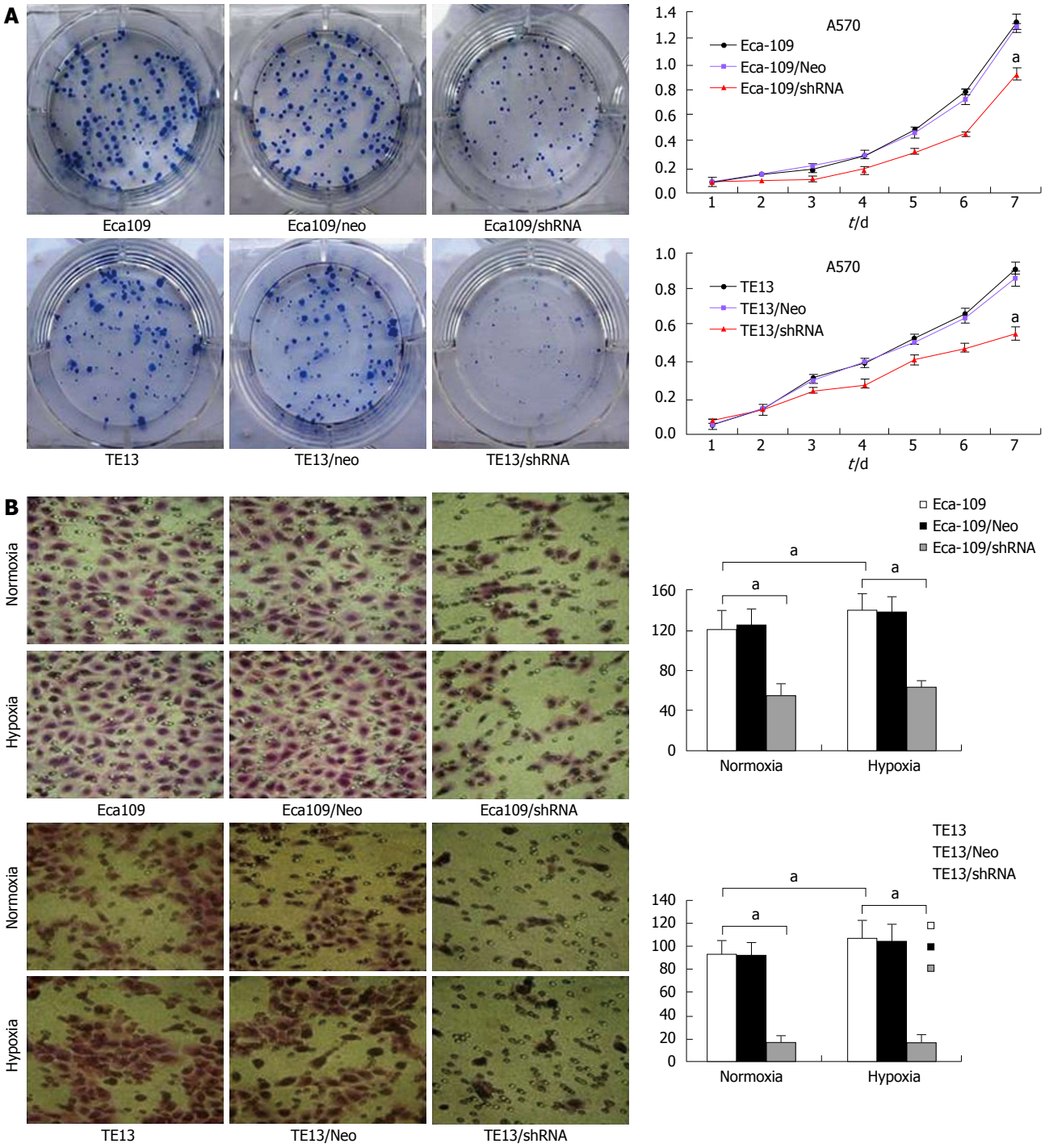

Figure 1 Effects of hypoxia inducible factor-1α knockdown on regulation of esophageal cancer cell viability and migration. A: MTT assay demonstrated that Eca109/HIF-1α shRNA and TE13/HIF-1α shRNA cells proliferated slower than normal Eca109 and TE13 cells or Eca109 HIF-1α Neo and TE13 HIF-1α Neo cells; B: Transwell migration assay demonstrated that Eca109/HIF-1α shRNA and TE13/HIF-1α shRNA cells invaded slower than normal Eca109 and TE13 cells or Eca109 HIF-1α Neo and TE13 HIF-1α Neo cells. Expression of HIF-1α and the four VM-related genes in TE13/shHIF and Eca109/shHIF cells under hypoxia *vs* under normoxia, $^{a}P < 0.05$. HIF: Hypoxia inducible factor.

expression was similar among the three groups. Quantitative PCR results for HIF-1α, EphA2, VE-cadherin and MMP2 in the Eca109 group were 1.0 ± 0.106, 1.0 ± 0.123, 1.0 ± 0.114 and 1.0 ± 0.172, respectively. In the Eca109/NeoHIF group, the PCR results for HIF-1α, EphA2, VE-cadherin and MMP2 were 1.103 ± 0.118, 1.078 ± 0.091, 0.937 ± 1.083 and 0.911 ± 1.106, respectively ($P > 0.05$). In the Eca109/shHIF group, the PCR results for HIF-1α, EphA2 and VE-cadherin were 0.684 ± 0.105, 0.713 ± 0.112 and 0.629 ± 0.094, respectively ($P < 0.05$) and for MMP2, it was 0.957 ± 0.162 ($P > 0.05$). These studies support the findings that knockdown of HIF-1α inhibits EphA2 and VE-cadherin expression in Eca109 cells *in vivo*.

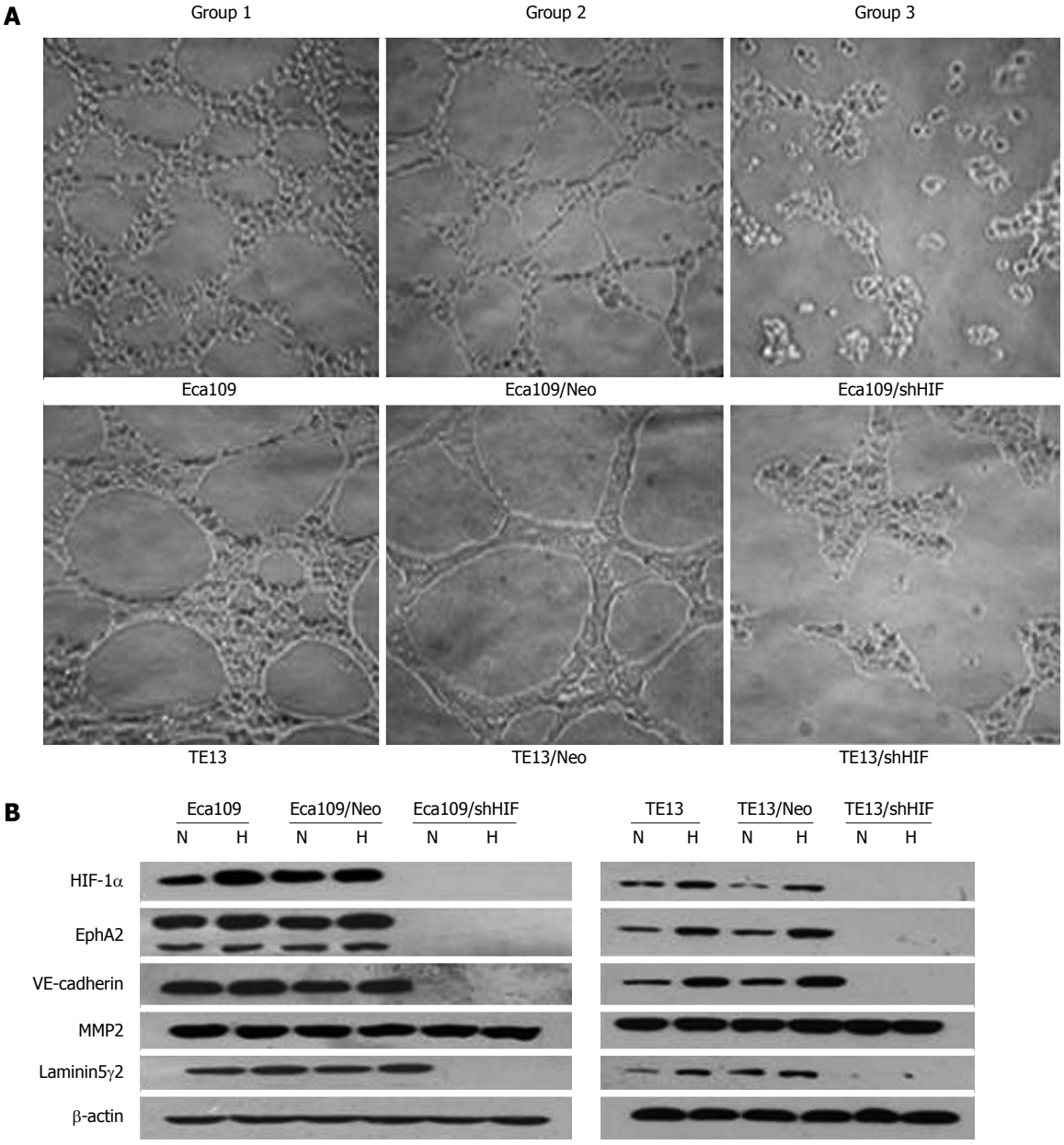

Figure 2 Knockdown of hypoxia inducible factor-1α inhibits vasculogenic mimicry formation and expression of vasculogenic mimicry-related genes *in vitro*. A: Effect of silencing HIF-1α on the formation of VM in ESCC cells (magnification × 200) (Group 1: Untransfected cells; Group 2: Cells transfected with empty vector; Group 3: Cells transfected with pGCsi-HIF3); Control group *vs* parental cells, $P < 0.05$; B: Expression of HIF-1α protein and VM-related genes in esophageal squamous cancer cells. Expression of HIF-1α and the four VM-related genes in TE13/shHIF and Eca109/shHIF cells under hypoxia *vs* under normoxia, $P < 0.05$. HPF: High-power field; HIF: Hypoxia inducible factor; VM: Vasculogenic mimicry; ESCC: Esophageal squamous cell carcinoma. N: Normoxia; H: Hypoxia.

Generation and characterization of ESCC cell clones stably expressing shVE-cadherin

Green fluorescence was observed by inverted fluorescence microscopy in Eca109/siVE-cad and TE13/siVE-cad cells (Figure 4A), which were G418-resistant monoclones. These results revealed that Eca109/siVE-cad and TE13/siVE-cad cells had stable expression of green fluorescent protein (*GFP*) gene inserted in plasmid pGCsi-VE-cad. Western blot analysis indicated that expression of VE-cadherin protein was inhibited > 90% compared to untransfected cells or cells transfected with VE-cad Neo control shRNA vector (Figure 4B).

Knockdown of VE-cadherin inhibits proliferation and promotes apoptosis of ESCC cells

MTT assay demonstrated that Eca109/shVE-cad and TE13/shVE-cad cells proliferated slower than normal Eca109 and TE13 cells or Eca109/Neo VE-cad and TE13/Neo VE-cad cells. On day 7, HIF-1α knockdown led to 36.8% ± 6.7% inhibition of Eca109 cell proliferation and 31.0% ± 6.2% inhibition of TE13 cell proliferation (Figure 5A). These results suggest that VE-cadherin could promote the proliferation of ESCC cells ($P < 0.05$).

The apoptosis rate in different groups of ESCC cells was determined by Annexin V-FITC as follows:

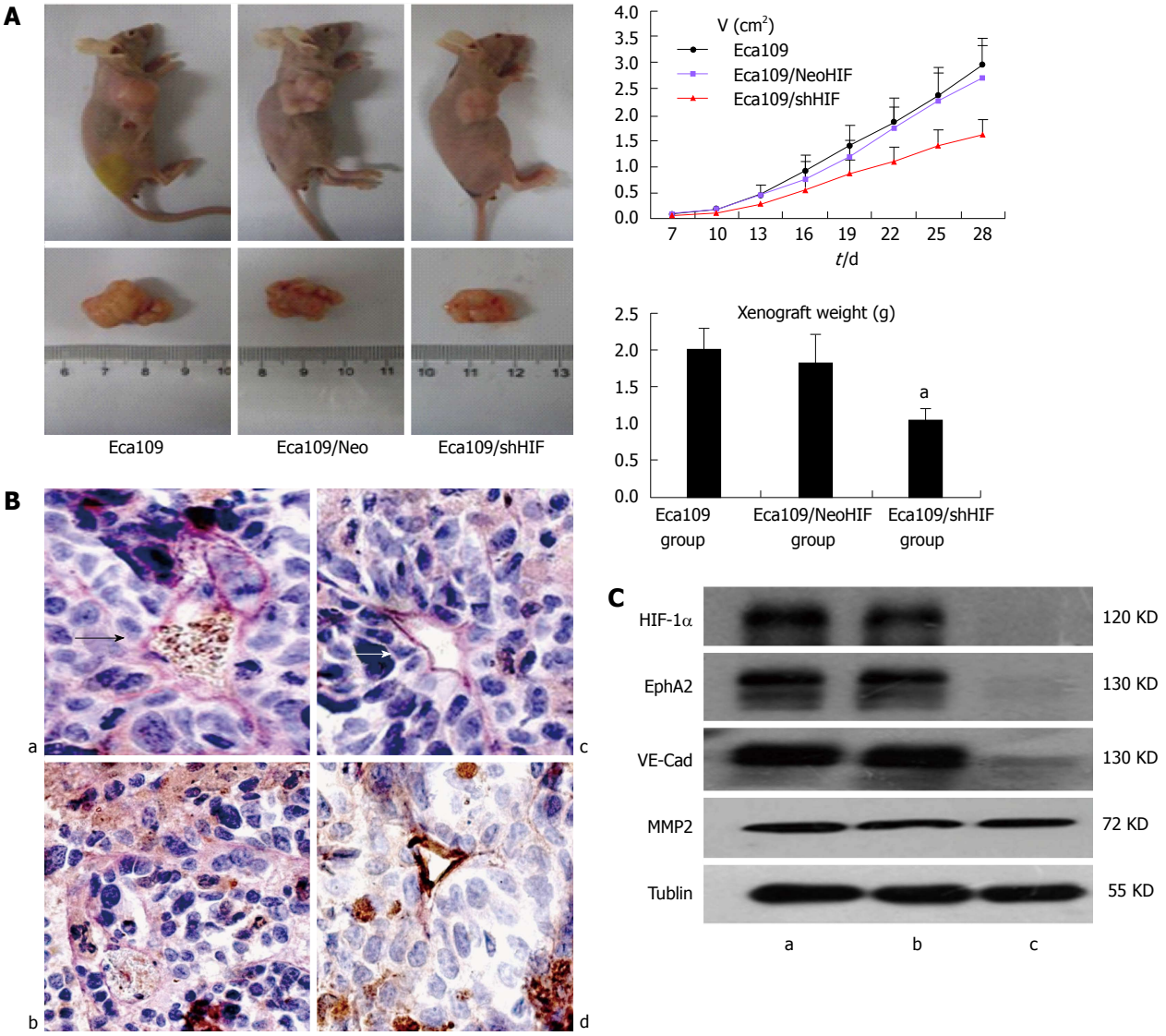

Figure 3 ***In vivo*** **effects of hypoxia inducible factor-1α knockdown on regulation of esophageal cancer cell xenograft formation and gene expression.** A: Tumor size curve and weight of xenograft (Eca109/shHIF-1α cells *vs* Eca109 cells and Eca109/HIF-1α Neo cells, $^{a}P < 0.05$); B: VM structure (arrow) in xenografts of three groups (a: Eca109 group; b: Eca109/shHIF group; c: Eca109/NeoHIF group; d: Normal vessels; arrow, VM structure); C: Western blot detected HIF-1α and EphA2, VE-cadherin and MMP2 expression (a: Eca109 group; b: Eca109/NeoHIF group; c: Eca109/shHIF group; Eca109/shHIF-1α cells *vs* Eca109 and Eca109 HIF-1α Neo control cells, $P < 0.05$). HIF: Hypoxia inducible factor; VM: Vasculogenic mimicry.

Eca109 6.77% ± 1.56%, Eca109/Neo 7.85% ± 1.47%, Eca109/shVE-cad 20.29% ± 5.23%; TE13 10.39% ± 3.08%, TE13/Neo 11.65% ± 3.79%, and TE13/shVE-cad 29.95% ± 7.39% (Figure 5B). Compared to control groups, the apoptosis rate in VE-cadherin knockdown cells was significantly increased in ESCC cells. These data indicate that VE-cadherin had an inhibitory effect on apoptosis of ESCC cells ($P < 0.05$)

shRNA against VE-cadherin inhibits VM formation and expression of VM-related genes in vitro

The channel-forming ability of Eca109/shVE-cad and TE13/shVE-cad cells *in vitro* was significantly inhibited compared with Eca109 and TE13 cells. This result demonstrated that the cyclic structure fractured and the number of tubular structures in the stable transfection group was markedly less than that in the control group (Figure 6A). The mRNA and protein expression of VE-cadherin, EphA2, and LN5γ2 was notably inhibited in Eca109/shVE-cad and TE13/shVE-cad cells under normoxic conditions ($P < 0.05$) and expression of HIF-1α did not change obviously (Figure 6B).

DISCUSSION

Hypoxia is a unique microenvironment in solid tumors, including ESCC[14]. The particular characteristics of the tumor microenvironment have the potential to strongly promote tumor growth, metastasis and angiogenesis and induce drug resistance. HIF-1α is a key transcription factor in tumor development and only accumulates in hypoxic tumors[15,16].

Under normoxic conditions, the HIF-1α subunit is rapidly degraded *via* the von Hippel-Lindau tumor

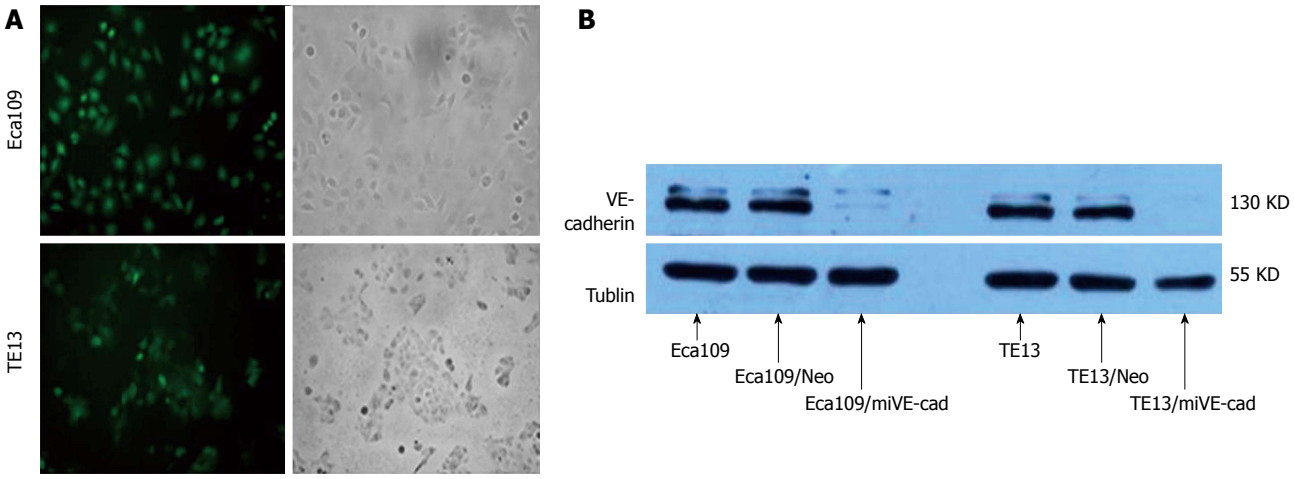

Figure 4 Identification of esophageal squamous cell carcinoma cells stably transfected with pGCsi-VE-cadherin. A: Expression of green fluorescent protein in two stably transfected ESCC cell lines (magnification × 200); B: Expression of VE-cadherin protein in the two ESCC cell lines. ESCC: Esophageal squamous cell carcinoma.

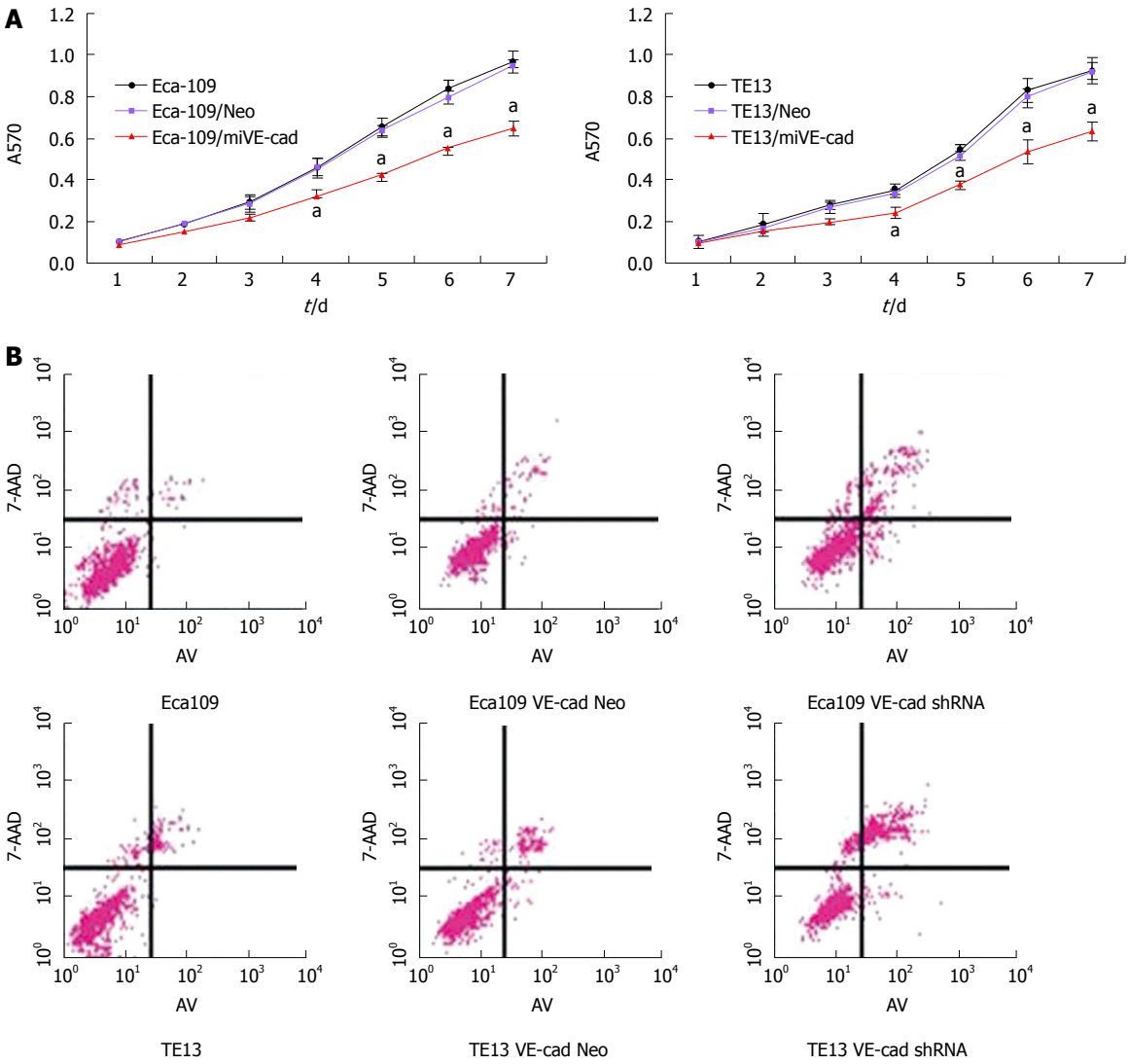

Figure 5 Knockdown of VE-cadherin inhibits proliferation and promotes apoptosis of esophageal squamous cell carcinoma cells. A: shRNA-mediated VE-cadherin knockdown inhibits the proliferation of ESCC cells. Eca109, TE13 and their derived stable cells were cultured under normoxic conditions and proliferation was determined by MTT assay; $^{a}P < 0.05$; B: shRNA-mediated VE-cadherin knockdown promoted apoptosis of ESCC cells. Eca109, TE13 and their derived stable cells were cultured under normoxic or hypoxic conditions and apoptosis was detected by Annexin V staining. ESCC: Esophageal squamous cell carcinoma.

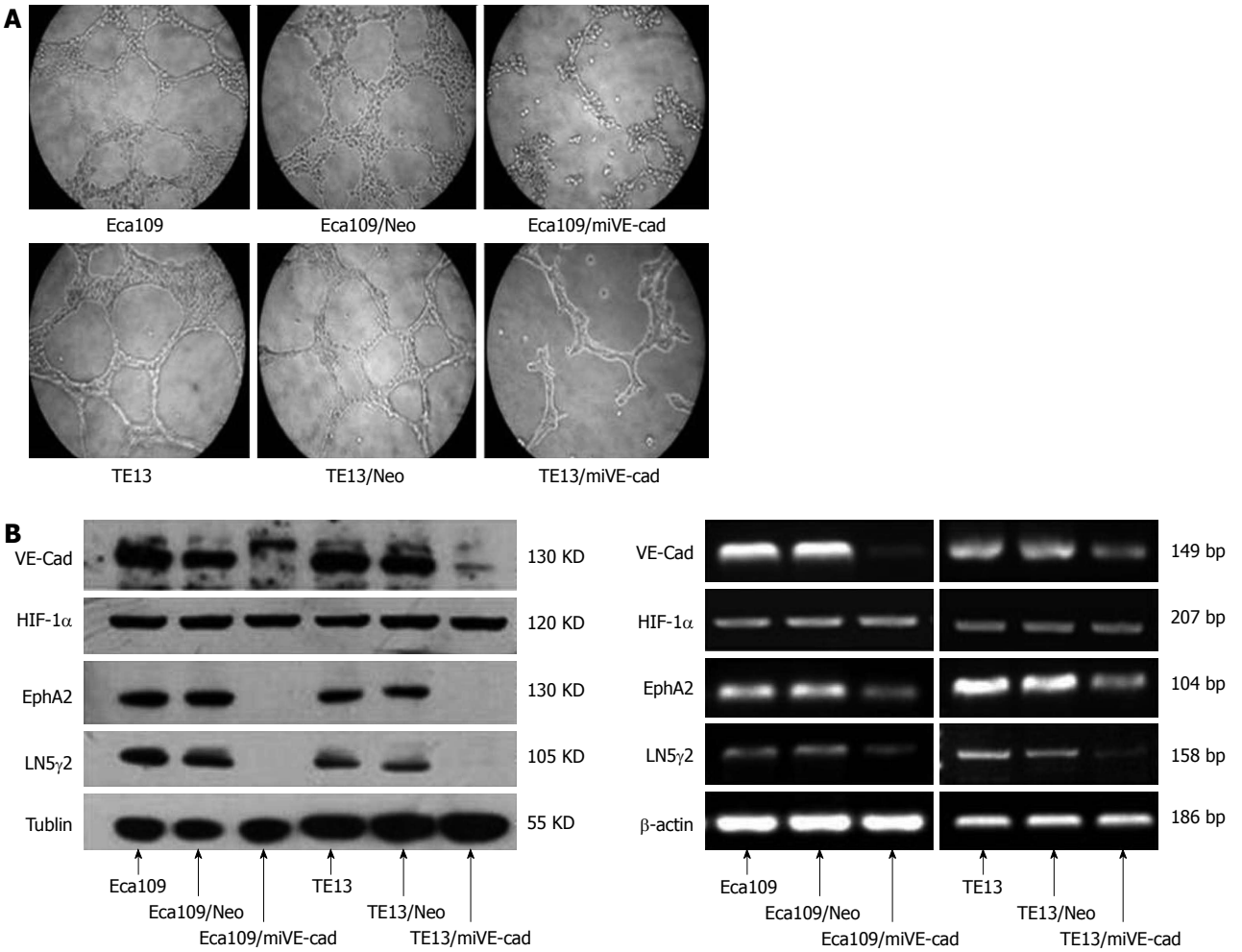

Figure 6 shRNA against VE-cadherin inhibits vasculogenic mimicry formation and expression of vasculogenic mimicry-related genes *in vitro*. A: Effect of silencing VE-cadherin on the formation of VM in ESCCs (magnification × 400); B: Protein and mRNA expression of VE-cadherin and VM-related genes in esophageal squamous cancer cells. ESCC: Esophageal squamous cell carcinoma; VM: Vasculogenic mimicry; VE-cad: VE-cadherin; LN5γ2: Laminin 5γ2; HIF: Hypoxia inducible factor; EphA2: EphrinA2.

suppressor gene product (pVHL)-mediated ubiquitin-proteasome pathway. Under hypoxic conditions, the translationally controlled tumor protein decreases the protein level of VHL and increases the protein level of HIF1α, therefore, HIF-1α subunit becomes stable[17,18]. Recent studies have demonstrated that the inhibition of HIF-1α expression could inhibit the development of lung, liver, stomach, and other tumors[19-21], which indicate that targeting HIF-1α appears to be effective for cancer treatment.

In our previous study, we found that HIF-1α expression significantly increased in hypoxia compared with normoxia and inhibition of HIF-1α expression by YC-1 could inhibit the development of ESCC[13]. In this study, we found that shRNA-mediated knockdown of HIF-1α inhibited cell proliferation and migration in both hypoxia and normoxia. We used a xenograft nude mouse model to prove that shRNA-mediated HIF-1α knockdown suppressed the tumorigenicity of ESCC cells *in vivo*. These results indicated that HIF-1α could become an important target for ESCC therapy.

In 1999, Maniotis *et al*[3] found that tissue sections from highly aggressive and metastatic melanoma contained patterned networks of interconnected loops of extracellular matrix, in which endothelial cells were not identified and tumor cells mimicked endothelial cells - a process called VM. Wang *et al*[22] reported that VM can supplement the function of blood vessels to transport nutrients and oxygen to maintain the growth of tumor cells in malignant tumors[23]. Studies revealed that the presence of VM was associated with the expression of MMP-2, MMP-14, EphA2 and LN5γ2 in medulloblastoma[22,24,25]. At present, VM has been found in many cancers, such as malignant melanoma, osteosarcoma, ovarian cancer and liver cancer[26-28]. VM is also present in ESCC[29]. As a new blood supply pathway, VM tends to exist in highly malignant tumor tissues[30] and patients with VM often have a poor prognosis[31-33]. Some molecular mechanisms of VM have been investigated, however, the exact mechanism and the key signaling pathway have not yet been elucidated.

In the present study, we found that VM exists in human ESCC and HIF-1α plays an important role in the development of VM in human ESCC, which has not been reported. Our data also showed that targeting HIF-1α or

VE-cadherin effectively inhibited formation of VM in human esophageal cancer cells, indicating that HIF-1α and VE-cadherin may be targeted for anti-ESCC therapy.

Recent publications have implied that development of VM in malignant carcinoma is closely related to VE-cadherin, EphA2, LN5γ2 and MMP-2[34-36]. Hess *et al*[37] brought forward the hypothesis that VE-cadherin and EphA2 activate the phosphoinositide 3-kinase and focal adhesion kinase (FAK) signaling pathways. These pathways further activate MT1-MMP, MMP-2 and then Laminin 5γ2 (LN5γ2) to promote the formation of VM[37]. However, in our study, the expression of VE-cadherin and EphA2 was inhibited after silencing of HIF-1α, while expression of MMP-2 was not significantly affected. The expression of *LN5γ2* gene was almost completely inhibited in Eca109/shHIF and TE13/shHIF cells. When VE-cadherin was silenced, expression of LN5γ2 and EphA2 was inhibited, while expression of HIF-1α was unchanged. These results suggested that VE-cadherin is the downstream gene of HIF-1α and regulates VM in ESCC through *EphA2* and *LN5γ2* genes but not *MMP2* gene.

In conclusion, the present study indicated that human ESCC cell lines could form vascular network-like structures, and HIF-1α plays an important role in the signal transduction pathway of the VM. The possible mechanism is that a hypoxic microenvironment causes greater expression of HIF-1α, and higher transcriptional activity of HIF-1α promotes the formation of VM by modulating *VE-cadherin*, *EphA2* and *LN5γ2* gene expression directly or indirectly to provide a blood supply to the tumor. Antiangiogenic drugs alone cannot completely block tumor blood supply. Thus, treatment strategies for carcinoma that presents with VM should take the latter into account, as well as targeting endothelium-dependent vessels.

COMMENTS

Background

The incidence of esophageal carcinoma is currently rising faster than any other cancer in the world, although the cause of this increase is largely unknown. Progression of this disease is associated with angiogenesis, a crucial event in tumor growth and metastasis.

Research frontiers

Vasculogenic mimicry (VM) is a common event in highly malignant tumor tissue, with the function of blood vessels to transport nutrients and oxygen to maintain the growth of tumor cells. Hypoxia inducible factor (HIF)-1α and VE-cadherin, the major endothelial adhesion molecules controlling blood vessel formation, are overexpressed in esophageal squamous cell carcinoma (ESCC). Whether hypoxia induces VM formation *via* upregulation of VE-cadherin by HIF-1α in ESCC has not been confirmed. In this study, we demonstrated that HIF-1α may upregulate the expression of VE-cadherin to accommodate the ability of forming VM in ESCC.

Innovations and breakthroughs

Recent reports have highlighted the importance of VM. VM is closely related to VE-cadherin, ephrin A2 (EphA2) and laminin 5γ2 (LN5γ2) in malignant carcinoma. This is believed to be the first study to report that VM also exists in human ESCC and HIF-1α plays an important role in VM development. This study also showed that targeting HIF-1α or VE-cadherin effectively inhibited formation of VM in human esophageal cancer cell lines.

Applications

By understanding how VE-cadherin is induced and by blocking its expression, this study may represent a future strategy for therapeutic intervention in the treatment of patients with ESCC.

Terminology

HIF is a key factor in regulating and promoting tumor progression. In this process, angiogenesis and VM may play an important role in helping tumors acquire more blood supply. VE-cadherin, EphA2 and LN5γ2 proteins are all involved in the process of VM.

Peer review

The authors discussed the role of HIF-1α in the formation of VM in ESCC. They showed that HIF-1α may increase expression levels of EphA2 and LN5γ2 by upregulating VE-cadherin expression in ESCC during formation of VM. The results are interesting and may also have implications in the treatment of malignant tumor disease.

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ORIGINAL ARTICAL

Protective effects of terminal ileostomy against bacterial translocation in a rat model of intestinal ischemia/reperfusion injury

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Abstract

AIM: To investigate the effects of terminal ileostomy on bacterial translocation (BT) and systemic inflammation after intestinal ischemia/reperfusion (I/R) injury in rats.

METHODS: Thirty-two rats were assigned to either the sham-operated group, I/R group, I/R + resection and anastomosis group, or the I/R + ileostomy group. The superior mesenteric artery was occluded for 60 min. After 4 h, tissue samples were collected for analysis. BT was assessed by bacteriologic cultures, intestinal permeability and serum levels of endotoxin; systemic inflammation was assessed by serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10, as well as by the activity of myeloperoxidase (MPO) and by intestinal histopathology.

RESULTS: Intestinal I/R injury not only caused morphologic damage to ileal mucosa, but also induced BT, increased MPO activity and promoted the release of TNF-α, IL-6, and IL-10 in serum. BT and ileal mucosa injuries were significantly improved and levels of TNF-α and IL-6 in serum were decreased in the I/R + ileostomy group compared with the I/R + resection and anastomosis group.

CONCLUSION: Terminal ileostomy can prevent the detrimental effects of intestinal I/R injury on BT, intestinal tissue, and inflammation.

Key words: Bacterial reflux; Bacterial translocation; Intestinal ischemia/reperfusion; Terminal ileostomy

Core tip: Few studies have evaluated the occurrence of bacterial cecoileal reflux. We performed ileostomy to block the route of bacterial cecoileal reflux after intestinal ischemia/reperfusion injury. Compared with resection and anastomosis, ileostomy could improve the bacterial translocation, ileal mucosal injuries and systemic inflammation. The results provide a theoretic basis for the choice of ileostomy and resection/anastomosis in clinical practice.

Lin ZL, Yu WK, Tan SJ, Duan KP, Dong Y, Bai XW, Xu L, Li N. Protective effects of terminal ileostomy against bacterial translocation in a rat model of intestinal ischemia/reperfusion injury. *World J Gastroenterol* 2014; 20(47): 17905-17913 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17905.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17905

INTRODUCTION

Bacterial translocation (BT) is an important factor affecting clinical prognosis when the body is under stress of any kind, such as trauma, burns or sepsis[1,2]. Previous studies have focused on the destruction of the intestinal mucosal barrier and the dysregulation of intestinal microbiota in disease[3-6], but have seldom focused on the importance of intestinal bacteria on reflux. Fritz *et al*[7] found that BT derives from the small bowel rather than from the colon. However, bacterial density in the small bowel is much lower than in the colon (10^4 and 10^7 bacteria/g in the jejunum and ileum, respectively, versus 10^{12} bacteria/g in the colon)[8]. The study by Berber *et al*[9] demonstrated that ileal bacterial counts increased approximately sevenfold following 24-h intestinal ischemia/reperfusion (I/R) injury. In addition, proliferation of the bacteria and the bacterial reflux from the cecum to the ileum could be important factors in small intestinal bacterial overgrowth, which is closely related to BT[10]. The occurrence of cecoileal reflux (CIR) during barium enema is a relatively common observation[11-13]. Perhaps due to difficulties in approaching the ileocecal junction, the most frequently adopted approach has been that of disregarding the importance of CIR and considering it as an irrelevant and common finding with no specific diagnostic meaning. Thus, there are few available data on the importance of CIR in BT.

Terminal ileostomy is one of the most common procedures in general surgery, and can disrupt the route of bacterial CIR. In the clinical situation, surgeons are often faced with a choice between ileostomy and primary anastomosis. Most previous studies comparing these two procedures were clinical trials, concerned mainly with morbidity and mortality[14-16]. Few studies have considered the influence of BT on the prognosis of patients. We postulate that ileostomy can interrupt the reflux of bacteria-rich colonic contents into the terminal ileum, thus alleviating bacterial overgrowth in the proximal intestine and improving BT in intestinal injury.

As a potentially important condition, intestinal I/R injury can be induced by traumatic, hemorrhagic or septic shock, acute mesenteric ischemia, severe burns, and some surgical procedures[17,18]. Previous studies suggested that intestinal I/R could induce injury of the intestinal mucosa following translocation of intestinal endotoxins and bacteria into blood, which may activate systemic inflammatory response syndrome and initiate multiple organ dysfunction syndrome[19]. An animal model of intestinal I/R injury is an established model for basic research, and intestinal ischemia caused by superior mesenteric artery (SMA) occlusion is stable and easy to repeat.

In this study, we investigated the protective effects of ileostomy against BT in a rat model of intestinal I/R injury and attempted to understand the intestinal and systemic inflammatory response following ileostomy and anastomosis, which could provide a theoretical basis for the choice of ileostomy and resection/anastomosis in clinical practice.

MATERIALS AND METHODS

Animals

Healthy adult male Sprague-Dawley rats (200-250 g) were obtained from Jinling Hospital, Nanjing, China. The rats were maintained in standard conditions with a 12 h light/dark cycle, controlled temperature (18 ± 4 ℃), and appropriate humidity (about 50%). To maintain the integrity of the intestinal mucosa, the animals had free access to standard chow and water for one week. This study was approved by the Animal Care and Use Committee of the Medical School of Nanjing University, and all animals received care in accordance with the guide of the committee.

Operative procedure

Before the experiment, rats were fasted overnight, but had free access to water. The rat model of intestinal I/R injury was established according to a method described previously[20]. The rats were anesthetized with an injection of ketamine hydrochloride (100 mg/kg; ip). During the surgery, the rats were allowed to breathe spontaneously. Body temperature was maintained at approximately 37 ℃ using a heating pad. To prevent dehydration, Ringer's lactate solution (10 mL/kg) was administered subcutaneously at the end of the operation. Prior to surgery, the abdomen was shaved and soaked twice with 10% povidine-iodine solution using sterile instruments. The abdomen was opened using a 4 cm midline incision, and the SMA was exposed. The rats were randomized into four groups: (1) sham-operated rats (sham-operated group; $n = 8$); (2) rats exposed to 60-min SMA occlusion followed by 4-h reperfusion (I/R group; $n = 8$); (3) rats exposed to I/R and then underwent partial intestinal resection and anastomosis (RA) (I/R + RA group; $n = 8$); and (4) rats exposed to I/R and then underwent terminal ileostomy (I/R + ileostomy group; $n = 8$) (Figure 1). In the sham-operated group, the SMA was isolated without occlusion. In the I/R, I/R + RA and I/R + ileostomy groups, the SMA was carefully isolated, and occluded at its origin with an atraumatic microvascular clamp for 60 min. The intestinal ischemia was confirmed by the loss of mesenteric pulsation and the intestines becoming pale. Reperfusion was confirmed by the return of mesenteric pulsation after removing the clamp. In the I/R group, the abdominal wall was immediately closed with a double-deck running suture. A 3 cm length of ileum at a point 2 cm proximal to the ileocecal valve was resected in the I/R + RA and I/R + ileostomy groups immediately after occluding the SMA. An end-to-end anastomosis was performed in the I/R + RA group, while in the I/R + ileostomy group, the end of the proximal ileum was brought out onto the abdominal wall as the ileostomy, and the distal end of the ileum was ligated. To avoid the bowel drying out, the abdominal cavity was irrigated with saline throughout the procedure.

Microbiologic analysis

All rats were anesthetized and euthanized after a 4-h re-

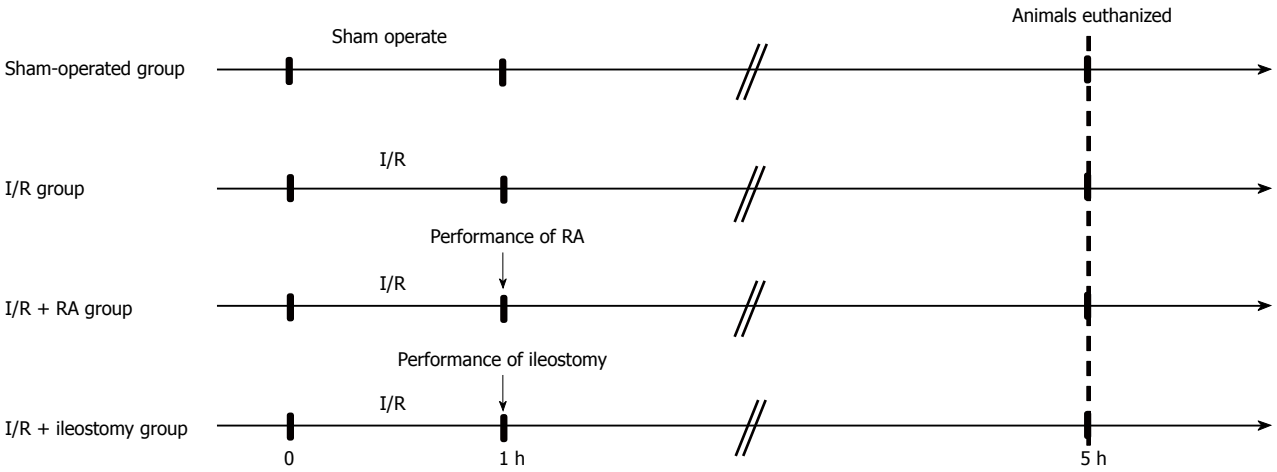

Figure 1 Experimental design. I/R: Ischemia/reperfusion; RA: Resection and anastomosis.

perfusion. The method used to perform microbiologic analysis was previously described[9]. Using an aseptic technique and sterile instruments, tissue samples from the liver, spleen, kidney, mesenteric lymph nodes (MLN), contents from the 10-cm terminal ileum and blood were taken for bacteriologic cultures. After being weighed, the ileal contents and tissue were stored in a sterile grinding tube. The tissues were homogenized in 1 mL saline. After diluting the homogenates, 0.1 mL dilutions were inoculated onto eosin methylene blue agar and blood agar, and incubated in ambient air for 24-48 h at 37 ℃. Blood (0.5 mL) samples were cultured in 5 mL of brain-heart infusion broth for seven days at 37 ℃. The cultures were checked daily, and sub-cultured on eosin methylene blue agar and blood agar plates. The number of colony-forming units/g of tissue homogenate was used to express the colonization.

Wet-to-dry weight ratio

Intestinal tissue samples (5 cm) were removed 15 cm proximal to the ileocecal valve and rinsed with saline. The wet weight of the intestine was determined and subsequently placed in a drying oven at 80 ℃ for 4 h. After desiccation, the tissue was weighed again to obtain the tissue dry weight. The ratio of the wet-to-dry (W/D) weight was calculated to provide an assessment of the extent of intestinal edema.

Assay of intestinal permeability

A modified method to assay the intestinal permeability was described previously[21]. At 4 h after reperfusion, we subjected additional rats from each group (n = 6 per group) to general anesthesia. After performing a midline laparotomy, a 5-cm-segment of distal ileum at a point 10 cm proximal to the ileocecal valve was isolated between silk ties. We injected a solution containing 25 mg of 4 kDa fluorescein isothiocyanate (FITC)-dextran diluted in 0.1 mL phosphate buffered saline into the enteric cavity. Then the bowel was put back in the abdominal cavity, and the abdomen was closed with a double-deck running suture. The animals were kept under general anesthesia. Thirty minutes after the injection of FITC-dextran, blood sample was collected by cardiac puncture and centrifuged at 10000 *g* for 10 min. Using a fluorescence spectrophotometer (F7000; Hitachi, Japan), the serous concentration of FITC-dextran was detected at excitation and emission wavelengths of 495 nm and 520 nm, respectively. By diluting serial serous concentrations of FITC-dextran, a standard curve was obtained.

Endotoxin and serum levels of TNF-α, IL-6 and IL-10 assays

After clotting for 60 min on ice, vena cava blood samples were centrifuged at 2500 × g for 10 min at 4 ℃. Sera were obtained and stored at -70 ℃. The serum levels of lipopolysaccharide were detected by colorimetric analysis for the limulus test (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's guidelines. The serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were measured by enzyme linked immunosorbent assay with commercially available kits (RD Systems, Minneapolis, MN, United States) according to the instructions of the manufacturer. Each reaction was performed twice.

Assay of myeloperoxidase activity

Myeloperoxidase (MPO) activity, which reflects polymorphonuclear neutrophil accumulation, was assessed by spectrophotometry. After weighing, intestinal tissue samples were homogenized on ice and MPO activity was measured quantitatively according to the manufacturer's instructions (Jiancheng Biologic Project Company, Nanjing, China). Data are expressed as U/g wet weight, where one unit of MPO activity is defined by the conversion of 1 mmol H_2O_2 to H_2O in one minute at 37 ℃.

Histopathology

Segments of terminal ileum were excised, fixed for 48 h in 10% formalin and then embedded in paraffin. Sections 5 μm thick were cut and placed on microscope slides, and stained by hematoxylin and eosin. Images at magnification × 20 were obtained using a Zeiss Image A1 light

Table 1 Intestinal mucosal damage grading score

Grade	Histologic characteristics
0	Normal mucosal villi
1	Development of subepithelial Gruenhagen's space, usually at the apex of the villus, often with capillary congestion
2	Extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria
3	Massive epithelial lifting with a few denuded villi
4	Denuded villi with exposed dilated capillaries
5	Digestion and disintegration of lamina propria, hemorrhage, and ulceration

microscope with Axiovision version4.5 software. Each slide was analyzed and reported by three pathologists who were all blinded to the source of the slides. A scale from 0 to 5 described by Chiu *et al*[22] was used to grade the intestinal mucosal lesions (Table 1).

Statistical analysis

Statistical analyses was performed with SPSS version 19.0 (IBM Corp., Armonk, NY, United States). Normally distributed data are shown as mean ± SD. χ^2 or Fisher's exact tests were used for the statistical evaluation of proportional comparisons for positive cultures of tissues. The remaining data were analyzed using one-way analysis of variance. Significant results were analyzed post hoc using the least significance difference test. Statistical significance was established as $P < 0.05$.

RESULTS

Bacterial translocation

During the experimental period, all the animals survived. In the I/R and I/R + RA groups, the incidence of BT to the liver, kidney, MLN and blood was significantly higher than in the sham-operated group ($P < 0.05$). However, the incidence of BT to the liver and MLN in the I/R + ileostomy group was significantly lower than in the I/R + RA group ($P < 0.05$). The I/R + ileostomy group and the sham-operated group only differed significantly for the incidence of BT to MLN ($P < 0.05$). In the I/R + ileostomy group, the counts of microorganisms cultured in the liver, spleen, kidney and MLN were significantly lower than in the I/R + RA group ($P < 0.05$; Table 2).

Ileal bacterial counts

Ileal bacterial counts in the I/R and I/R +RA groups were significantly higher than those in the sham-operated group ($P < 0.01$). Compared with the I/R + RA group, ileostomy significantly reduced the ileal bacterial counts after intestinal I/R injury ($P < 0.01$; Table 3).

W/D weight ratio

The intestinal W/D weight ratios in the I/R, I/R + RA and I/R + ileostomy groups were significantly higher than in the sham-operated group ($P < 0.01$). There were no significant differences in the intestinal W/D weight ratio between the I/R group and the I/R + RA group. The intestinal W/D weight ratio in I/R + ileostomy group was significantly lower than in the I/R + RA group ($P < 0.01$) (Figure 2).

Assay of intestinal permeability

Compared with the sham-operated group, the FITC-dextran levels detected in the serum were significantly higher after intestinal I/R injury ($P < 0.01$). Furthermore, RA or ileostomy did not significantly increase the amount of circulating FITC-dextran compared with the I/R group (Figure 3). These results indicate that RA or ileostomy did not increase intestinal permeability in I/R rats.

Endotoxin assays

Intestinal I/R injury significantly increased the endogenous endotoxin levels compared with the sham-operated group. However, the endotoxin levels in the I/R + ileostomy were significantly lower than in the I/R + RA group ($P < 0.01$; Figure 4).

Assay of TNF-α, IL-6 and IL-10 in serum

Compared with the sham-operated group, I/R significantly increased serum levels of the pro-inflammatory TNF-α ($P < 0.01$). RA after I/R injury increased the level of TNF-α in serum compared with I/R only rats, but was not significantly different. However, ileostomy after I/R injury did not increase serum TNF-α levels, which were significantly lower when compared with the I/R + RA group ($P < 0.01$). Similarly, the increases in serum IL-6 levels in the I/R, I/R + RA and I/R + ileostomy groups were significant as compared with sham-operated animals ($P < 0.01$). Ileostomy after I/R injury decreased serum IL-6 levels, which were significantly lower when compared with the I/R + RA group ($P < 0.01$). Serum IL-10 levels, as an indicator of anti-inflammatory response, were significantly higher in the I/R, I/R + RA and I/R + ileostomy groups than in the sham-operated animals ($P < 0.01$). Serum IL-10 levels in I/R, I/R + RA and I/R + ileostomy groups were not different (Figure 5).

Assay of MPO activity

Compared with the sham-operated group, MPO activity in the I/R and I/R + RA groups was significantly higher ($P < 0.05$). MPO activity in the I/R + ileostomy group was significantly lower than in the I/R group ($P < 0.05$), but higher than in the sham-operated group ($P < 0.05$) (Figure 6).

Histopathology

As shown in Figure 7, intestinal tissue segments from the sham-operated group showed normal morphology. Multiple erosions and massive infiltration of inflammatory cells in the lamina propria were observed after intestinal I/R injury. After I/R injury, damage to crypt cells and shortening of villi were typical histologic appearances. After intestinal I/R injury, atrophied mucosal tissue and leukocyte infiltration could be observed.

The Chiu's histopathology score was lowest in the

Table 2 Incidence and number of bacterial translocations in tissue and blood samples

Group (n = 8)	Liver		Spleen		Kidney		MLN		Blood	
	Incidence	CFU	Incidence	CFU	Incidence	CFU	Incidence	CFU	Incidence	CFU
Sham	0/8	0 ± 0	0/8	0 ± 0	0/8	0 ± 0	0/8	0 ± 0	0/8	0 ± 0
I/R	6/8[a]	841 ± 206.19[a]	4/8	33 ± 13.39[a]	5/8[a]	261 ± 93.66[a]	7/8[a]	49750 ± 10592.70[a]	5/8[a]	36 ± 12.25[a]
I/R + RA	7/8[a]	1316 ± 236.22[a]	6/8[a]	61 ± 15.42[a]	6/8[a]	375 ± 88.74[a]	8/8[a]	64750 ± 8645.29[a]	5/8[a]	38 ± 11.84[a]
I/R + ileostomy	2/8[b]	171 ± 112.78[b]	2/8	12 ± 7.69[b]	3/8	68 ± 33.42[b]	3/8[ab]	4850 ± 2510.19[b]	2/8	13 ± 5.40

CFU: Colony-forming units; I/R: Ischemia/reperfusion; MLN: Mesenteric lymph nodes; RA: Resection and anastomosis; [a]P < 0.05 *vs* sham; [b]P < 0.05 *vs* I/R + RA.

Table 3 Ileal bacterial counts

Groups	Ileal bacterial count (CFU/g)
Sham	82375 ± 10390.48
I/R	528750 ± 204341.28[a]
I/R + RA	671250 ± 60280.22[a]
I/R + ileostomy	177750 ± 21987.62[b]

CFU: Colony-forming units; I/R: Ischemia/reperfusion; RA: Resection and anastomosis. [a]P < 0.01 *vs* sham; [b]P < 0.01 *vs* I/R + RA.

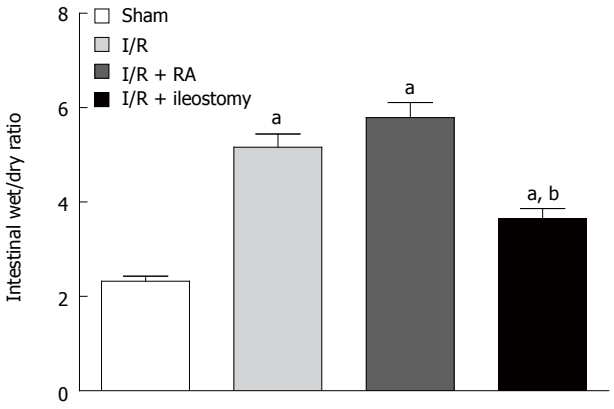

Figure 2 Wet-to-dry weight ratios. Data are expressed as mean ± SD. [a]P < 0.01 *vs* sham; [b]P < 0.01 *vs* I/R + RA. I/R: Ischemia/reperfusion; RA: Resection and anastomosis.

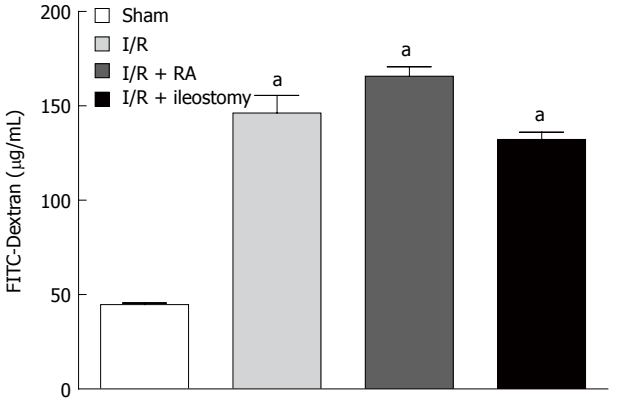

Figure 3 Fluorescein isothiocyanate-dextran levels in serum. Data are expressed as mean ± SD. [a]P < 0.01 *vs* sham. FITC: Fluorescein isothiocyanate; I/R: Ischemia/reperfusion; RA: Resection and anastomosis.

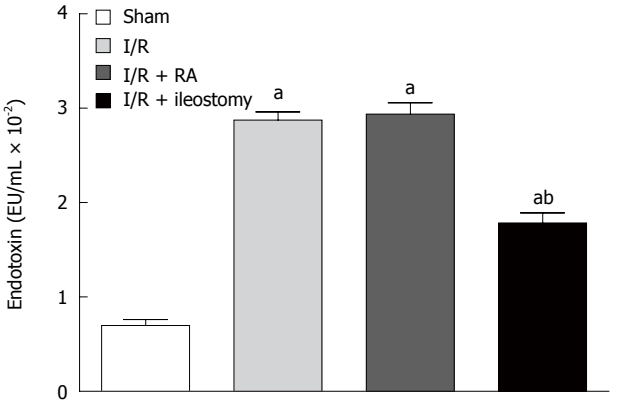

Figure 4 Endotoxin levels in serum. Data are expressed as mean ± SD. [a]P < 0.01 *vs* sham; [b]P < 0.01 *vs* I/R + RA. I/R: Ischemia/reperfusion; RA: Resection and anastomosis.

sham-operated group, and was highest in the I/R + RA group (P < 0.01). Although there was no significant difference in scores among the I/R, I/R + RA and I/R + ileostomy groups, the score in the I/R + ileostomy group was lower than in the I/R and I/R + RA groups (Figure 8).

DISCUSSION

This is the first study to investigate BT in relation to ileostomy and intestinal resection/anastomosis, and the potential role of ileostomy in ameliorating BT and systemic inflammation in a rat model of intestinal I/R injury.

Physiologically, the intestinal barrier provides physical integrity and mucosal immunity[23]. Intestinal I/R injury can lead to the destruction of endothelial and epithelial cells and the breakdown of mucosal integrity, which results in increased intestinal permeability and dysfunction of the intestinal barrier[24]. Consequently indigenous intestinal bacteria translocate to other organs such as the blood, spleen, liver and MLN, leading to sepsis and multiple organ dysfunction syndrome[24]. In the present study, we found that construction of an ileostomy immediately after occlusion of the SMA significantly decreased the incidence of BT to the spleen, liver, kidney and MLN. Ileostomy also significantly reduced ileal bacterial overgrowth. Thus, ileostomy seems to have protective effects on the intestinal tract. Although it is not known how the ileostomy prevents translocation, we can hypothesize that the prevention of contamination by CIR appears to be related to the protective effect of ileostomy. It has been

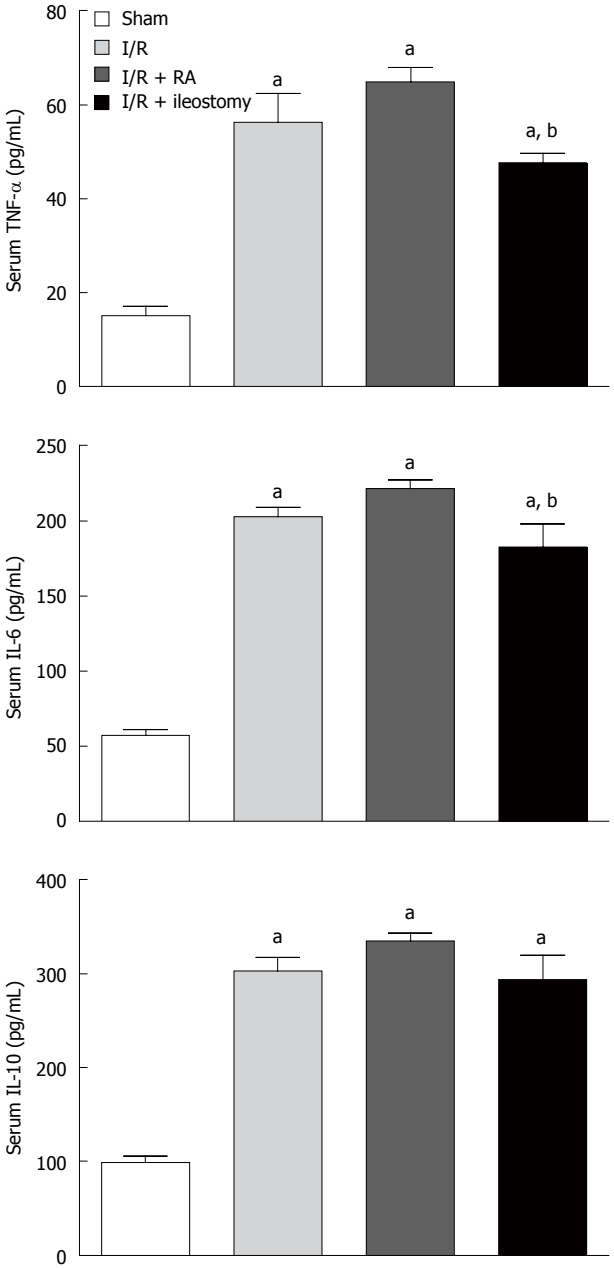

Figure 5 Serum levels of tumor necrosis factor tumor necrosis factor-α, -6 and IL-10 following intestinal ischemia/reperfusion injury in rats. Data are expressed as mean ± SD. $^{a}P < 0.01$ *vs* sham; $^{b}P < 0.01$ *vs* I/R + RA group. IL: Interleukin; TNF-α: Tumor necrosis factor-α; I/R: Ischemia/reperfusion; RA: Resection and anastomosis.

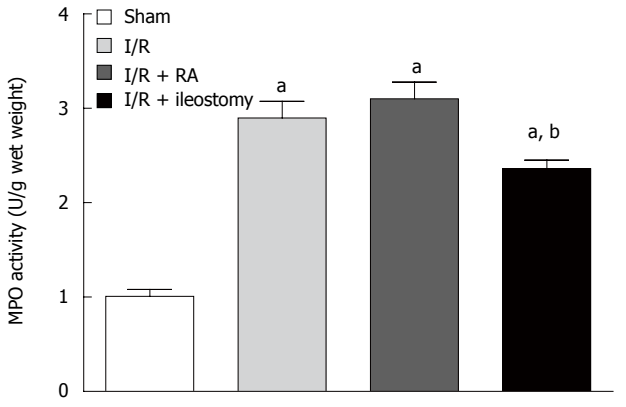

Figure 6 Myeloperoxidase activity in distal ileal tissue. Data are expressed as mean ± SD. $^{a}P < 0.05$ *vs* sham; $^{b}P < 0.05$ *vs* I/R + RA group. I/R: Ischemia/reperfusion; RA: Resection and anastomosis; MPO: Myeloperoxidase.

shown by previous studies that intestinal I/R could not only cause injury to mucosa, but also delay the gastrointestinal transit time[25-27]. At the same time, accumulating experimental evidence has shown the occurrence of ileal contamination due to reflux in experimental models or after surgical intervention in the ileocecal valve[28-30]. The occurrence of CIR during barium enema is a common observation[11-13]. These are refluxes of varying intensity that oscillate from small to large that can fill long ileal segments. The results of our experiment suggest that the protective effects of ileostomy on intestinal BT may be partly due to the prevention of contamination by CIR.

As the initial BT process, attachment of bacteria to the enterocyte can mediate the production of cytokines, resulting in an inflammatory response. Following the passage of bacteria through the epithelial barrier, pro-inflammatory factors are excessively released, resulting in the destruction of intestinal barrier integrity and further BT[31]. The present study suggests that the serum levels of TNF-α and IL-6, the pro-inflammatory cytokines, increased after 4-h intestinal I/R injury. These results are consistent with previous studies demonstrating that intestinal I/R injury has a large effect on the release of cytokines[31]. It has been demonstrated that increased mortality in animal models is relevant to the increase in TNF-α and IL-6 levels after intestinal I/R injury[32,33]. The degree of tissue injury and mortality after intestinal I/R injury is determined by the balance between TNF-α and IL-10[34]. We found that, when compared with RA, ileostomy did not increase the levels of IL-6 and TNF-α in serum after intestinal I/R injury. This may be due to the decreased incidence of BT observed in rats treated with ileostomy.

In addition to the indigenous bacterial translocation, the endotoxic translocation may also induce the dysfunction of other organs as a result of changes in intestinal function. Endotoxin could potently stimulate the release of cytokines, such as IL-6 and TNF-α. These mediators of inflammation play an important role in the pathogenesis of systemic inflammatory response syndrome[35] and multiple organ dysfunction syndrome[36]. This study demonstrated that ileostomy after intestinal I/R injury significantly decreased the serum level of endotoxin. We reasoned that the protective effect of ileostomy appears to be related to its ability to prevent the reflux of endotoxin-rich colonic contents into the ileum.

As an indicator of neutrophil accumulation, MPO activity is increased in intestinal I/R injury[9,37]. In this study, the reduction in neutrophil accumulation, which was shown by the significantly decreased MPO activity in the intestine of ileostomy-treated rats, seems to be a consequence of preventing BT. In the course of intestinal I/R injury, neutrophils play an important role[38]. Af-

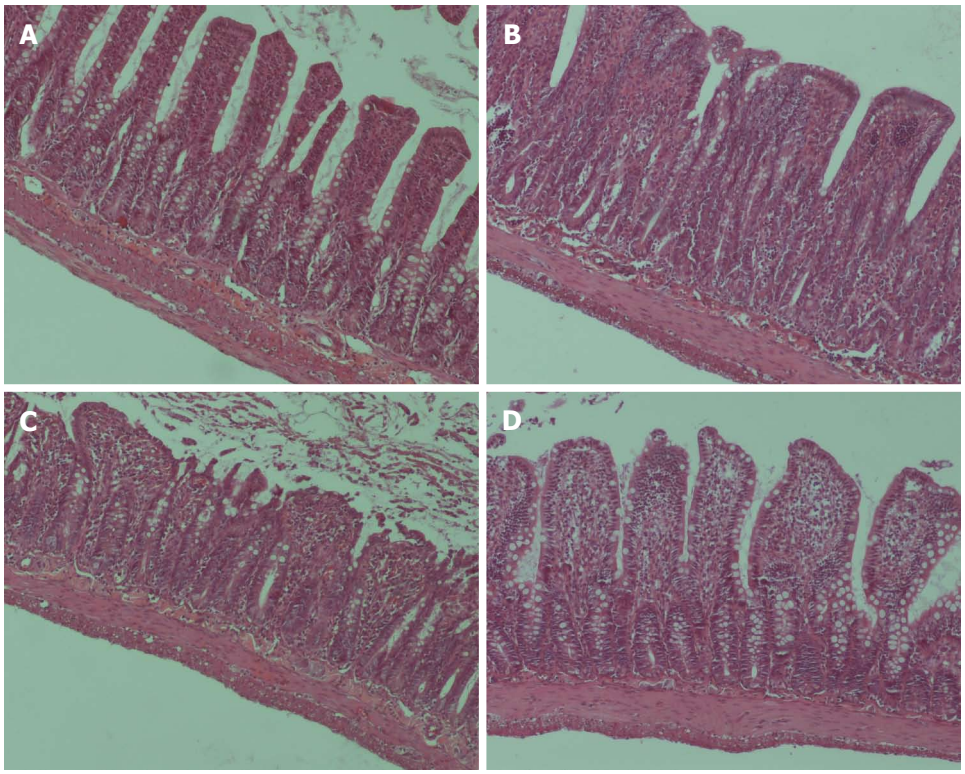

Figure 7 Light microscopic evaluation of the distal ileum. Hematoxylin and eosin staining in distal ileum (magnification × 40) in the A: sham-operated group, showing an intact mucosal barrier with normal lamina propria; B: Intestinal ischemia/reperfusion (I/R) injury resulted in acute mucosal damage; C: Resection and anastomosis did not ameliorate the mucosal damage caused by I/R injury; D: I/R + ileostomy resulted in an apparently intact mucosal barrier, though the epithelial cells appeared shrunken.

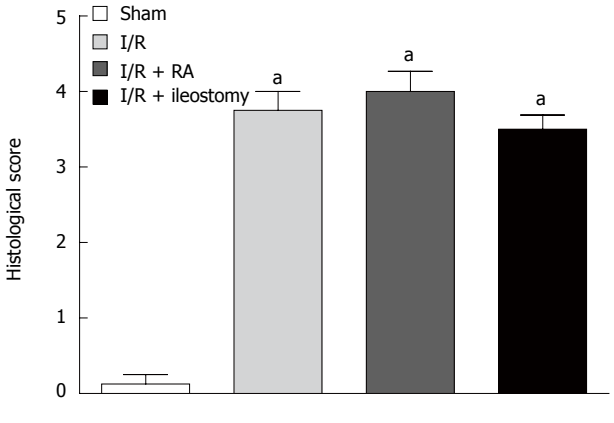

Figure 8 Histologic injury score of the intestines. Data are expressed as mean ± SD. $^{a}P < 0.01$ *vs* sham. I/R: Ischemia/reperfusion; RA: Resection and anastomosis.

ter intestinal I/R injury, mediators such as bacteria and cytokines appears to stimulate the systemic activation of neutrophils[38]. Further inflammation and oxidative damage was then promoted by these activated neutrophils.

Our data showed that the construction of a terminal ileostomy after intestinal I/R injury partially reversed tissue damage, and decreased intestinal W/D weight ratio and the histologic injury score. In addition to the prevention of ileal contamination by CIR, possible explanations for these results might be that the terminal ileostomy promotes the ileal clearing function after intestinal I/R injury. However, due to a lack of appropriate studies, terminal ileum clearing delay in individuals with CIR cannot be confirmed, and it is not possible to determine whether such a delay may be bacteria-specific or extend to other contents. As noted in gastroesophageal reflux disease genesis[39,40], further studies focusing on the pathogenic implications of inadequate clearing of individuals with CIR are desirable. If this delay is confirmed, it could lead to prolonged exposure of the ileum to bacteria and metabolic products originating in the colon[41,42], resulting in harmful consequences to body balance.

Demonstrating the effects of interrupting CIR on BT was the main purpose of this research. However, the time when CIR appears after intestinal I/R injury is difficult to determine. Therefore, further studies are needed to define the optimal time to perform a terminal ileostomy. Another limitation of this study is that we did not use exact methodology to confirm the CIR. Isotope- or fluorescent-labeled bacteria could be used to conduct further experiments.

In conclusion, this study suggests that ileostomy ameliorates the detrimental effects of intestinal I/R injury on the intestine and systemic inflammation in rats. We speculate that bacterial CIR plays an important role in BT, and the beneficial effects of ileostomy, such as improved BT and systemic inflammation, are attributed mainly to the

interruption of CIR.

COMMENTS

Background

Bacterial translocation (BT) is an important factor affecting clinical prognosis when the body is under stress of any kind, including trauma, burns or sepsis. Previous studies paid little attention to the importance of the reflux of intestinal bacteria. Terminal ileostomy is one of the most common procedures in general surgery, which can block the route of bacterial cecoileal reflux (CIR). In clinical practice, surgeons often face the choice between ileostomy and resection/anastomosis. In this study, the authors investigated the protective effect of ileostomy against BT in a rat model of intestinal ischemia/reperfusion (I/R) injury to provide a theoretic basis for this surgery.

Research frontiers

The authors demonstrated that ileostomy could prevent the detrimental effects of intestinal I/R injury on the intestinal tissue and systemic inflammation in a rat model of superior mesenteric artery occlusion. They speculate that bacterial CIR plays an important role in BT, and the beneficial effects of ileostomy, such as the improved BT and systemic inflammatory, are attributed mainly to the interception of CIR.

Innovations and breakthroughs

Recent reports have highlighted the importance of BT in intestinal I/R injury in rats. Few studies have described the bacterial CIR during intestinal I/R injury. This is believed to be the first study to report that ileostomy could prevent the detrimental effects of intestinal I/R injury on BT, intestinal tissue, and inflammation. This study also provided theoretic gist for the choice of ileostomy and resection/anastomosis in clinical practice.

Applications

Based on the findings in this study that ileostomy can prevent the detrimental effects of intestinal I/R injury on the intestinal tissue and systemic inflammation in a rat model of superior mesenteric artery occlusion, ileostomy may be performed as a more suitable therapeutic option than resection/anastomosis to prevent BT after intestinal I/R injury, which requires exploratory laparotomy.

Terminology

CIR is a relatively common observation in barium enema. The refluxes are of varying intensity that oscillate from small to large, which can fill long ileal segments. The hypothesis of CIR innocuousness seems, however, rather implausible as it would differ from the behavior of refluxes in other topographies of the digestive system, where they exhibit a high complication potential, generating such frequent and repercussive diseases as the gastroesophageal reflux disease. What is actually observed is that, perhaps due to the difficulties in approaching the ileocecal junction, the most frequently adopted conduct has been that of disregarding the importance of CIR and considering it as an irrelevant and common finding with no specific diagnostic meaning.

Peer review

The study of Lin and coworkers clearly shows how ileostomy may contribute to preventing the detrimental effects of ligating the superior mesenteric artery. The paper is well designed, the conclusions are supported by specific data, and its length and bibliography are appropriate.

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ORIGINAL ARTICLE

MicroRNA-185 regulates expression of lipid metabolism genes and improves insulin sensitivity in mice with non-alcoholic fatty liver disease

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Author contributions: Zhan XR and Liu XM designed the research; Wang XC, Li XY and Yu JJ performed the research; Wang XC and Li XY analyzed the data; Wang XC wrote the paper.

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Abstract

AIM: To assess the regulatory effect of microRNA-185 (miR-185) on lipid metabolism and the insulin signalling pathway in human HepG2 hepatocytes and a high-fat diet mouse model.

METHODS: Quantitative reverse transcription-polymerase chain reaction was used to assess the mRNA levels of lipogenic genes after loss or gain of miR-185. In addition, the amounts of insulin signalling intermediates were determined after transfection of HepG2 cells with pre-miR-185.

RESULTS: MiR-185 levels decreased in a time- and dose-dependent manner in response to palmitic acid in human HepG2 hepatocytes. Transfection of HepG2 cells with miR-185 significantly decreased the mRNA levels of fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, sterol-regulatory element binding protein-2, and sterol-regulatory element binding protein-1c, whereas inhibition of miR-185 using an anti-miR-185 oligonucleotide produced the opposite effect in HepG2 cells. In a high-fat diet mouse model, the accumulation of lipids was significantly improved after treatment with miR-185, compared with control animals. Induction of miR-185 enhanced the insulin signalling pathway by up-regulating the insulin-receptor substrate-2.

CONCLUSION: These findings suggest that miR-185 plays an important role in regulating fatty-acid metabolism and cholesterol homeostasis in hepatocytes, as well as in improving insulin sensitivity, both *in vitro* and *in vivo*.

Key words: MiR-185; Insulin signalling pathway; Lipid metabolism; Non-alcoholic fatty liver disease

Core tip: Our study presents important information on the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and insulin resistance. We used a bioinformatics method to identify microRNAs potentially involved in the regulation of lipid metabolism and insulin signalling. We chose microRNA-185 (miR-185) to validate *in vitro* and *in vivo* in regard to regulation of lipid metabolism gene expression and blockade of the insulin signalling pathway. We found that overexpression of miR-185 improved insulin sensitivity and reduced liver steatosis in an NAFLD animal model. No previous studies have reported the regulatory effect of miR-185 on the insulin signalling pathway. MiR-185 might be useful in the design of therapeutic strategies for treating NAFLD and insulin resistance.

Wang XC, Zhan XR, Li XY, Yu JJ, Liu XM. MicroRNA-185 regulates expression of lipid metabolism genes and improves insulin

sensitivity in mice with non-alcoholic fatty liver disease. *World J Gastroenterol* 2014; 20(47): 17914-17923 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17914.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17914

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a complex metabolic disease characterised by accumulation of triglycerides and free cholesterol in the liver. This condition is caused by an imbalance in lipid metabolism pathways involved in triacylglycerol delivery, synthesis, export or oxidation[1]. Indeed, the liver plays a central role in modulating lipid metabolism and homeostasis, and a disruption of lipid metabolism and insulin signalling is frequently closely associated with NAFLD[2-4]. Therefore, identification of the regulators that control lipid metabolism and insulin resistance is critical.

We previously analysed microarray expression profiles in NAFLD and insulin resistance in an effort to identify such regulators, using samples from eight-week-old C57bl/6 mice fed a high-fat diet (HFD)[5] and hepatic c-Jun amino-terminal kinase 1 (JNK1) knockout DIO mice[6], respectively. From a pool of thousands of genes, we identified 21 genes that were co-differentially expressed in NAFLD and insulin resistance groups, compared with normal liver tissue, using significance analysis of microarray data (Wang, Zhan *et al*, unpublished data). TargetScan algorithms[7] (http://www.targetscan.org) were used to predict miRNAs potentially targeting the 21 co-differentially expressed genes. Ultimately, microRNA-185 (miR-185) was predicted to modulate seven of the 21 co-differentially expressed genes. The putative interactions between miR-185 and these genes indicate its potential impact on NAFLD pathogenesis and insulin resistance. However, the exact role of miR-185 in the regulation of lipid metabolism and the insulin signalling pathway is yet to be determined. In addition, the accuracy of the bioinformatics prediction needs to be experimentally confirmed.

Hepatic insulin resistance plays a fundamental role in both carbohydrate and lipid metabolism[8]. Indeed, insulin-mediated activation of insulin-receptor substrate (IRS) and Akt2 is indispensable for glucose and lipid metabolism[9]. Recent evidence has emerged that the phosphatidylinositide 3-kinase (PI3K)/Akt signalling pathway activates the sterol-regulatory element-binding proteins (SREBPs), which results in dramatic effects on lipogenic gene expression, inducing steatosis[10]. It has been shown that SREBP-1 activation contributes to fatty acid and lipid accumulation, while SREBP2 is mainly responsible for cholesterol metabolism[11,12]. Recently, Li *et al*[13] have confirmed the regulatory role of miR-185 in lipogenesis and cholesterogenesis through targeting SREBP1 and SREBP2 in prostate cancer cells.

To date, no report is available on the role of miR-185 in lipid metabolism and the insulin signalling pathway in mice and HepG2 cells. Therefore, this study aimed to characterize the effect of miR-185 on NAFLD and insulin resistance *in vivo* and *in vitro*.

We herein validated the regulatory effect of miR-185 on lipid metabolism and the insulin signalling pathway. Indeed, we demonstrated that miR-185 is actively involved in the regulation of lipid metabolism and insulin sensitivity, using both *in vitro* assays and a C57BL/6 mouse model of NAFLD. These findings further reveal the critical role of miR-185 in various cellular processes, and provide a basis for the use of this potent regulator in the design of novel and better molecular targets for the treatment of NALFD and, to some extent, diabetes.

MATERIALS AND METHODS

Culture and transfection of HepG2 cells

HepG2 cells were cultured in DMEM containing 10% foetal bovine serum, 1% of an antibiotic cocktail containing penicillin (10000 U/mL) and streptomycin (10000 μg/mL), 1% non-essential amino acid solution, 1% *L*-glutamine and 5.0 mmol/L glucose, in a humidified environment with 5% CO_2 at 37 ℃. MiR-185, control microRNA, anti-miR-185 and control antisense oligonucleotides (ASOs) were synthesized by RiboBio Co., Ltd. (Guangzhou, China); the oligonucleotides were transfected into HepG2 cells using the Lipofectin transfection reagent (Invitrogen, United States), according to the manufacturer's instructions.

Animals and diets

The animal study was approved by the ethics committee of Harbin Medical University (Harbin, Heilongjiang, China) and experiments were conducted according to the National Institutes of Health guidelines for humane treatment of laboratory animals. Eight-week-old male C57Bl/6 mice were obtained from Harbin Medical University Laboratories (Harbin, Heilongjiang, China) and housed with a 12 h light/dark cycle, allowing free access to water and pellet chow. To assess miR-185 expression levels, C57BL/6 mice were divided into two groups, fed a normal laboratory diet and an HFD (30% fat, 15% protein, 45% carbohydrate, and 1.15% cholesterol), respectively, for 12 wk. At 4, 8 and 12 wk, liver tissues were harvested for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) experiments respectively.

For miR-185 treatment, 20 eight-week-old male C57BL/6 mice were fed an HFD for 12 wk. Then, half of the mice (n = 10) were intravenously injected with 20 mg/kg miR-185 while the other half, used as controls (n = 10), were administered equal amounts of the control microRNA weekly for 8 wk. Mouse body weights were recorded weekly.

Quantitative real-time RT-PCR

To determine whether miR-185 acts as a regulator in lipid metabolism, we first quantitated alterations in miR-185 expression in response to palmitic acid (PA) stimulation. The HepG2 cells were treated with 1.0×10^{-4} or

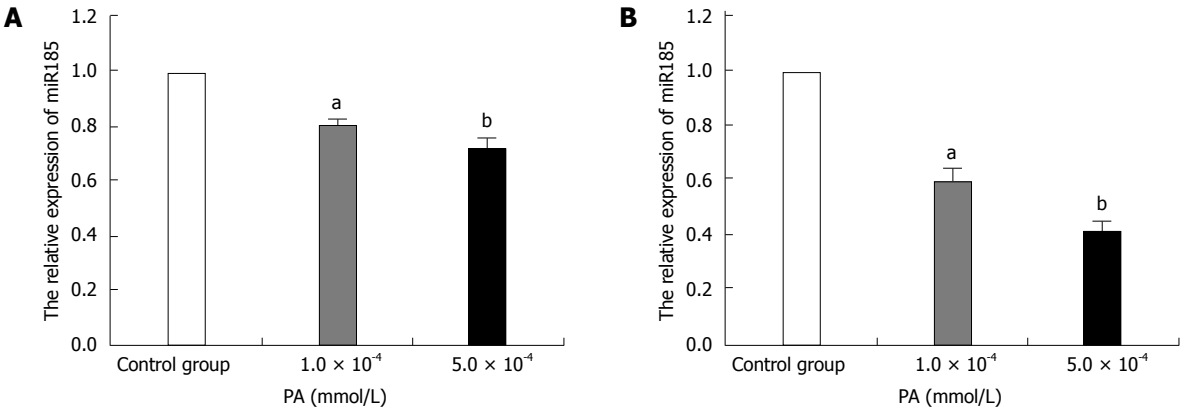

Figure 1 MicroRNA-185 expression levels in palmitic acid-treated HepG2 cells. HepG2 cells were treated with 1.0×10^{-4} or 5.0×10^{-4} mmol/L PA; qRT-PCR detected microRNA-185 (miR-185) levels after 24-h (A) or 48-h (B) incubation. RNU6-2 was used as an internal control for miR-185. Data are mean ± SEM from three separate experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the normal control group. PA: Palmitic acid; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

Table 1 The primer sequences

SREBP1
5'-ACC CCT GTG TTA GGC TAC CCC AGC CCT C-3'
5'-TCT CCG CAT CTA CGA CCA GTG GGA CTG T-3'
427 bp
SREBP2
5'-TCA AAC TCA GCT GCA ACA ACA GAC GGT A-3'
5'-AAT GAT ATT ATG GGT TGT CCG CCT TTC T-3'
592 bp
GAPDH
5'-TGC CAA ATA TGA TGA CAT CAA GAA GGT G-3'
5'-GTC ATA CCA GGA AAT GAG CTT GAC AAA G-3'
190 bp
FAS
5'-CTG GCT ACC TGA GCA TAG TGT GGA AGA C-3'
5'-TGC AGT GTG TAC AGC TTC TGC CTG TGG G-3'
544 bp
HMGCR
5'-ACA ATA AGA TCT GTG GTT GGA ATT ATG A-3'
5'-CCT AAA ATT GCC ATT CCA CGA GCA ATA T-3'
376 bp

SREBP1: Sterol-regulatory element-binding proteins-1; SREBP2: Sterol-regulatory element-binding proteins-2; GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase; FAS: Fatty acid synthase; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase.

5.0×10^{-4} mmol/L PA, and qRT-PCR was used to assess miR-185 levels after 24 h and 48 h of culture.

Total RNA was isolated from the liver tissue or cultured cells using a High Pure miRNA Isolation Kit (5080576001, ROCHE, Germany) according to the manufacturer's instructions. Then cDNA was synthesized using a miRcute miRNA cDNA kit (KR201-01, Invitrogen, United States). qRT-PCR was performed on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, United States).

To assess mRNA levels of SREBP1, SREBP2, fatty acid synthase (FAS), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), total RNA was extracted and purified using Trizol (Invitrogen, United States) and the RNeasy Mini kit (Qiagen), respectively, according to the manufacturers' instructions. The purified RNA was treated with DNase I and reverse transcribed using the MMLV Reverse Transcriptase First Strand cDNA Synthesis Kit (Invitrogen, United States). All reactions were performed in triplicate, and RNU6-2 or β-actin was selected as an internal control for normalization. Relative fold changes in gene expression were determined by the $2^{-\Delta\Delta Ct}$ method[14]. The primer sequences used for RT-PCR are listed in Table 1.

Assessment of fatty acid oxidation and sterol synthesis rates in HepG2 cells

HepG2 cells were cultured in 12-well plates and transfected with miR-185 (20 nmol/L), anti–miR-185 (40 nmol/L), or negative controls (con-miR and con-anti-miR). After 48 h, the fatty acid oxidation rate was evaluated by quantifying the oxidation of [1-^{14}C] oleate into $^{14}CO_2$, as previously described (Yu *et al*, 1997). The sterol synthesis rate was estimated by measuring the amounts of [^{14}C] acetate incorporated into cellular sterols, as described previously (Ettinger *et al*, 1994). Each experiment was performed in triplicate.

Western blot

Forty-eight hours after transfection, cells were washed in phosphate-buffered saline (PBS) and lysed with 300 μL RIPA lysis buffer (Solarbio, Beijing, China) containing a protease inhibitor mixture (Roche Applied Science, Germany). Proteins were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk and probed with primary antibodies raised against insulin receptor substrate (IRS) 1 (Abcam, United States), IRS-2 (Santa Cruz, United States), AKT2 (Santa Cruz, United States), PI3K (Santa Cruz, United States), and β-actin (Sigma, United States). After three washes with TBS-T, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, United States). The signals were visualised with an ECL kit (GE

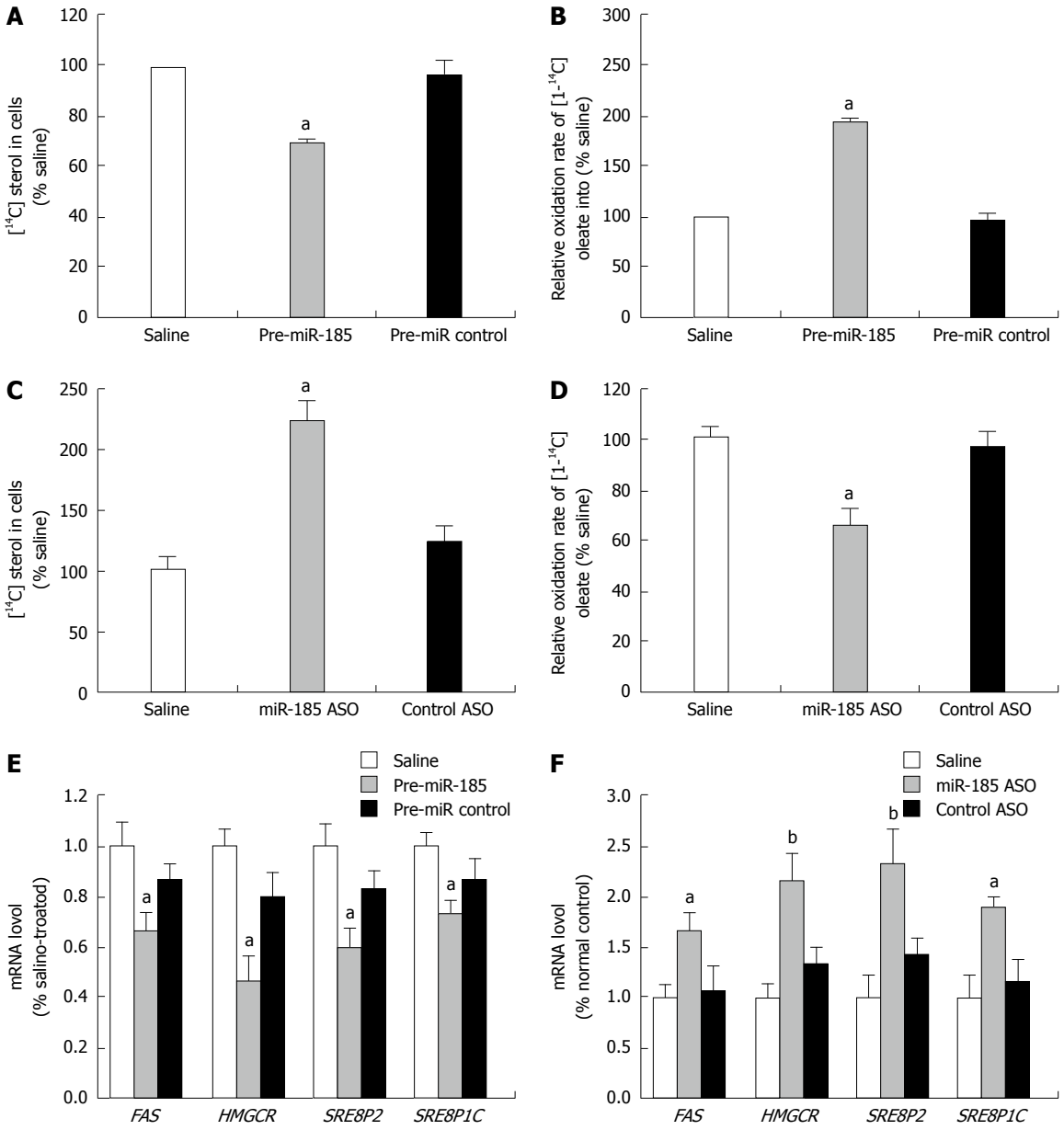

Figure 2 Effect of miR-185 on lipid metabolism in HepG2 cells. HepG2 cells were transfected with pre-miR-185 or anti-miR-185 (an antisense oligonucleotide against miR-185), and fatty acid oxidation and sterol synthesis rates were determined. A: The sterol synthesis rate was determined by the amount of [^{14}C] acetate incorporated into HepG2 cell sterols after transfection with pre-miR-185; B: The fatty acid oxidation rate was measured by the oxidation of [1-^{14}C] oleate into $^{14}CO_2$ after transfection with pre-miR-185; C: Sterol synthesis rates in HepG2 cells after transfection with miR-185 inhibitors; D: Fatty acid oxidation rates in HepG2 cells after transfection with miR-185 inhibitors; E: Quantitative reverse transcription-PCR was used to assess the mRNA levels of key lipid metabolism-associated genes in HepG2 cells after overexpression of miR-185; F: mRNA levels of lipid metabolism-associated genes after miR-185 inhibition. Data are mean ± SEM from three separate experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the normal control group. miR-185: microRNA-185; ASO: Antisense oligonucleotide; SREBP-1C: Sterol-regulatory element-binding proteins 1C; SREBP-2: Sterol-regulatory element-binding proteins 2; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase.

Healthcare, CT, United States).

Evaluation of clinical chemistry parameters

Plasma concentrations of total cholesterol (CHOL), triglycerides (TG), and alanine aminotransferase (ALT) were determined after the last injection using spectrophotometric assay kits (Sigma-Aldrich, St. Louis, MO, United States) according to the manufacturer's instructions.

Intraperitoneal insulin tolerance test

Mice were submitted to 8 h fasting and administered insulin by intraperitoneal injection. Then, plasma insulin levels were measured by ELISA and blood glucose levels (in tail vein blood) were assessed at 0, 15, 30, 60, and 120 min after insulin (1.0 U/kg body weight) treatment using a blood glucose meter (One Touch Ultra, Lifescan, United States) according to the manufacturer's instructions.

Histological analysis

To evaluate the effect of miR-185 on hepatic steatosis, liver tissues were harvested from mice after the last injection of miR-185, fixed in 10% formalin solution, and

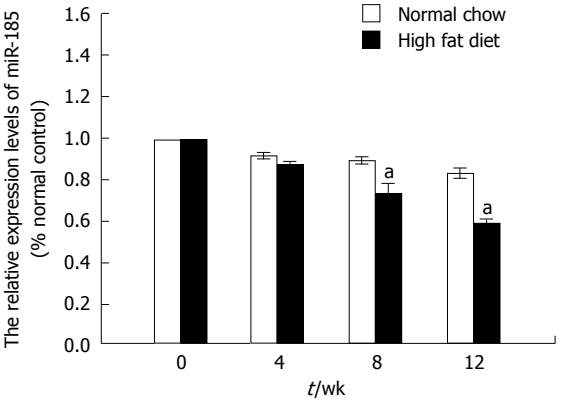

Figure 3 MicroRNA-185 expression levels in C57BL/6J mice fed a high fat diet. C57BL/6J mice were fed a normal chow or a high fat diet for 12 wk and liver tissues were harvested at 4, 8, and 12 wk. The miR-185 levels were determined by quantitative reverse transcription-PCR (n = 10). Data are mean ± SEM from three separate experiments. $^{a}P < 0.05$ *vs* the normal control group. miR-185: microRNA-185.

embedded in paraffin. The sections were stained with hematoxylin and eosin according to standard protocols.

Statistical analysis

Statistical analyses were carried out using SPSS statistical software version 12.0 (SPSS Inc., Chicago, IL, United States). Data are expressed as mean ± SD. Student's t-test and one way analysis of variance (ANOVA) were used to compare the differences between two or among more than two groups, respectively. $P < 0.05$ and $P < 0.01$ were considered statistically significant and highly significant, respectively.

RESULTS

MiR-185 expression is regulated by palmitic acid in HepG2 cells

In the presence of palmitic acid, miR-185 expression levels decreased by approximately 20% and approximately 35% after treatment with 1.0×10^{-4} mmol/L PA ($P <$ 0 .01) and 5.0×10^{-4} mmol/L PA ($P < 0.05$), respectively, after 24-h incubation compared with control cells (Figure 1A). This effect was increased after 48-h incubation, with miR-185 expression decreasing by approximately 42% (1.0×10^{-4} mmol/L PA, $P < 0.01$) and 60% (5.0×10^{-4} mmol/L PA, $P < 0.05$) compared with the control group (Figure 1B). These data suggested that miR-185 expression levels decreased in a time- and dose-dependent manner in response to PA in HepG2 cells.

Effect of miR-185 on lipid metabolism in HepG2 cells

To evaluate the modulatory effect of miR-185 on lipid metabolism, HepG2 cells were transfected with pre-miR-185 or anti-miR-185, and fatty acid oxidation and sterol synthesis rates were determined. Compared with control cells, HepG2 cells overexpressing miR-185 displayed a stark decrease in the sterol synthesis rate of approximately 30% ($P < 0.01$; Figure 2A) and an increased fatty acid oxidation rate (1.9-fold, $P < 0.01$; Figure 2B). Inhibition of miR-185 by anti-miR-185 resulted in a 2.2-fold increase in the sterol synthesis rate ($P < 0.01$, Figure 2C) and approximately 38% decrease in the fatty acid oxidation rates ($P < 0.01$, Figure 2D) compared with controls.

Since multiple studies have shown that FAS, HMGCR, SREBP2, and SREBP1c are strongly associated with lipid metabolism, the effect of miR-185 on these lipid metabolism-associated genes was assessed. Gene expression levels of *FAS*, *HMGCR*, *SREBP2*, and *SREBP1c* were measured after induction or inhibition of miR-185. qRT-PCR data revealed dramatically reduced expression of *FAS*, *HMGCR*, *SREBP2*, and *SREBP1c* after miR-185 overexpression in HepG2 cells (Figure 2E). Conversely, inhibition of miR-185 in HepG2 cells resulted in increased *FAS*, *HMGCR*, *SREBP2*, and *SREBP1c* mRNA levels (Figure 2F). These findings indicated that miR-185 regulates fatty acid metabolism and cholesterol homeostasis by suppressing the expression of lipogenic genes in hepatocytes.

Effect of a high fat diet on miR-185 expression in C57BL/6J mice

The *in vitro* data described above prompted us to further investigate the role of miR-185 in regulating lipid metabolism *in vivo*. C57BL/6J mice were fed a normal chow or a high fat diet for 12 wk, and liver tissues were harvested after sacrifice at weeks 4, 8, and 12 for qRT-PCR experiments. MiR-185 expression was decreased by approximately 22% at 8 wk ($P < 0.01$) and further declined (approximately 30%) at 12 wk ($P < 0.01$) compared with normal controls. In agreement with the *in vitro* data, miR-185 expression declined in the HFD group in a time-dependent manner (Figure 3).

Overexpression of miR-185 reduces liver steatosis and improves insulin sensitivity in vivo

To elucidate the regulatory effect of miR-185 on insulin sensitivity and lipid metabolism, c57BL/6 mice were fed a high-fat diet for 12 wk, then treated intraperitoneally weekly with miR-185 (20 mg/kg) for 8 wk. To determine insulin sensitivity, an ITT was performed after the last miR-185 administration. The results showed significantly lower plasma glucose concentrations in the HFD + miR-185 group compared with the HFD + con-miR group at all time points after injection of 1U/kg insulin (Figure 4A).

The effect of miR-185 overexpression on NAFLD was also assessed. After the 8-wk treatment regimen, no significant difference was observed between the HFD + con-miR and HFD + miR-185 groups in terms of mouse body weights (Figure 4B); TG, CHOL and ALT levels decreased after miR-185 treatment compared with the HFD + con-miR group (Figure 4C-E). In addition, HE staining showed enlarged cells with hepatocellular ballooning in the HFD + con-miR group. Finally, lipid accumulation decreased significantly after miR-185 treatment (Figure

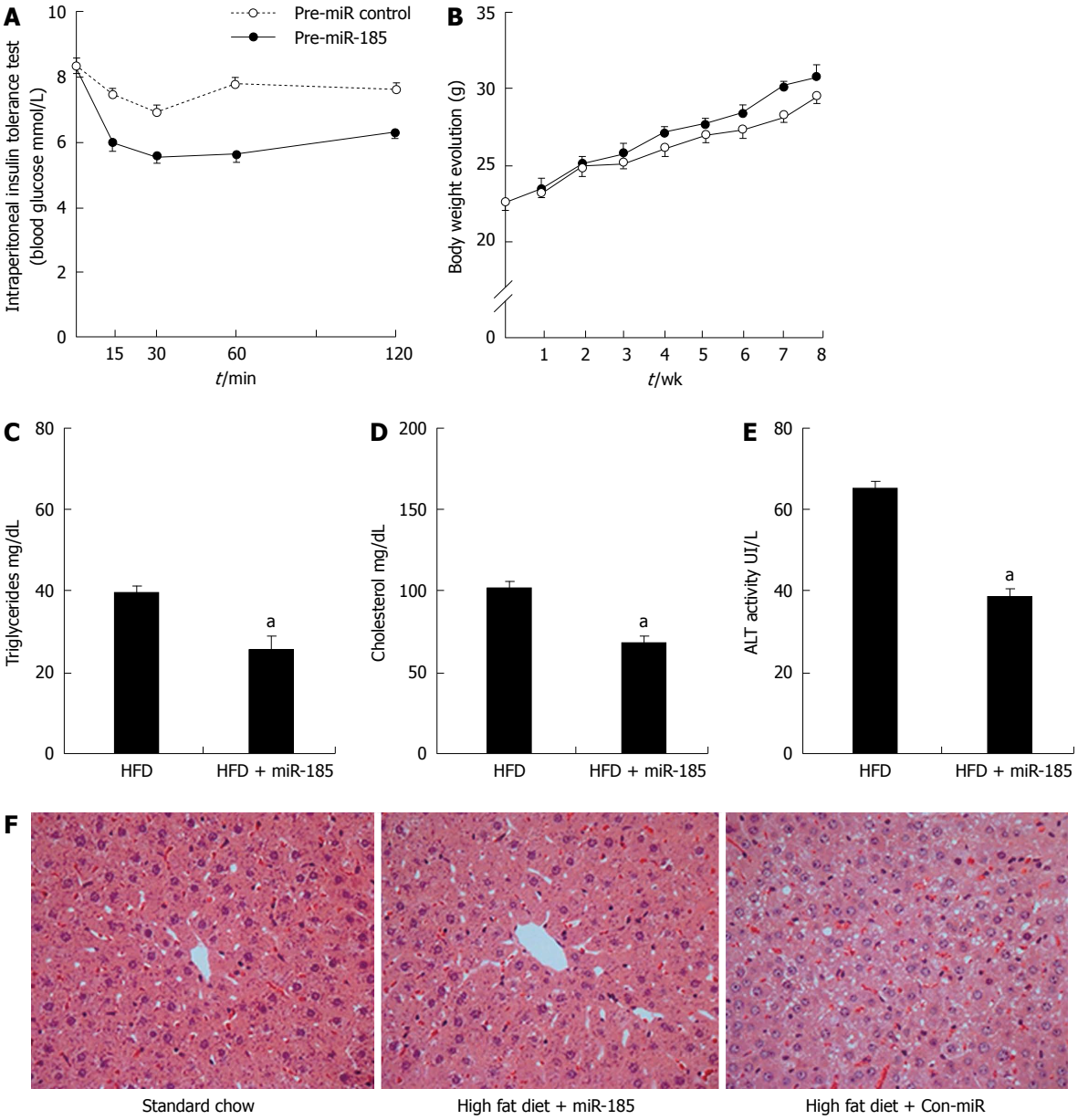

Figure 4 Overexpression of microRNA-185 improves insulin sensitivity and reduces liver steatosis. Eight-week-old male C57BL/6 mice were fed a high-fat diet for 12 wk. One half of the mice (n = 10) were injected with miR-185 (20 mg/kg body weight) and the control group (n = 10) received injections of control microRNA for 8 wk. A: ITT was performed after the last injection, and mice were submitted to 8 h fasting before intraperitoneal administration of insulin; B: Body weights were recorded weekly during the treatment period; C-E: Triglycerides, cholesterol and alanine aminotransferase levels were assessed after the last injection; F: At the end of the treatment, liver sections were stained by haematoxylin and eosin stain to assess lipid accumulation (magnification × 400). Where applicable, data are mean ± SEM from three separate experiments, n = 10, $^{a}P < 0.05$ *vs* the normal control group. HFD: High-fat diet.

4F). These data suggested that miR-185 overexpression alleviates liver fat content in C57BL/6 mice.

MiR-185 regulates the insulin signalling pathway in vitro

To explore whether miR-185 regulates the insulin signalling pathway, we assessed the expression of key pathway components (IRS-1, IRS-2, PI3K and Akt2) and the phosphorylation of Akt2 and PI3K after transfection of HepG2 cells with pre-miR-185. As shown in Figure 5A, IRS-2 expression was significantly elevated (3-fold) after miR-185 overexpression, whereas the expression levels of IRS-1, PI3K and Akt2 were unchanged. Moreover, both pPI3K and pAkt2 were markedly increased (Figure 5B), suggesting that miR-185 promotes the PI3K/Akt2 pathway by inducing IRS-2 expression rather than IRS-1.

DISCUSSION

Insulin resistance is critical in the pathogenesis of NAFLD, and both pathologies are frequently present concurrently[15-17]. Our previous work has focused on the molecular interaction between NAFLD and insulin resistance. Although the pathogenesis of NAFLD is not entirely understood, the critical role of miRNAs as major regulators

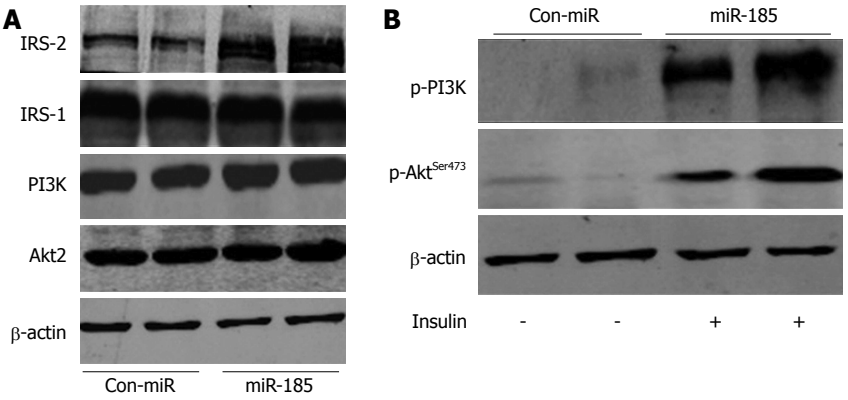

Figure 5 MicroRNA-185 regulates the insulin signalling pathway in HepG2 cells. A: The HepG2 cells were transfected with pre-miR-185 or pre-miR control and, after 48 h, the protein expression levels of insulin receptor substrate (IRS)-1, IRS-2, phosphatidylinositide 3-kinase (PI3K) and Akt2 were detected by Western blot; B: Effect of miR-185 on Akt2 and PI3K phosphorylation. miR-185: microRNA-185.

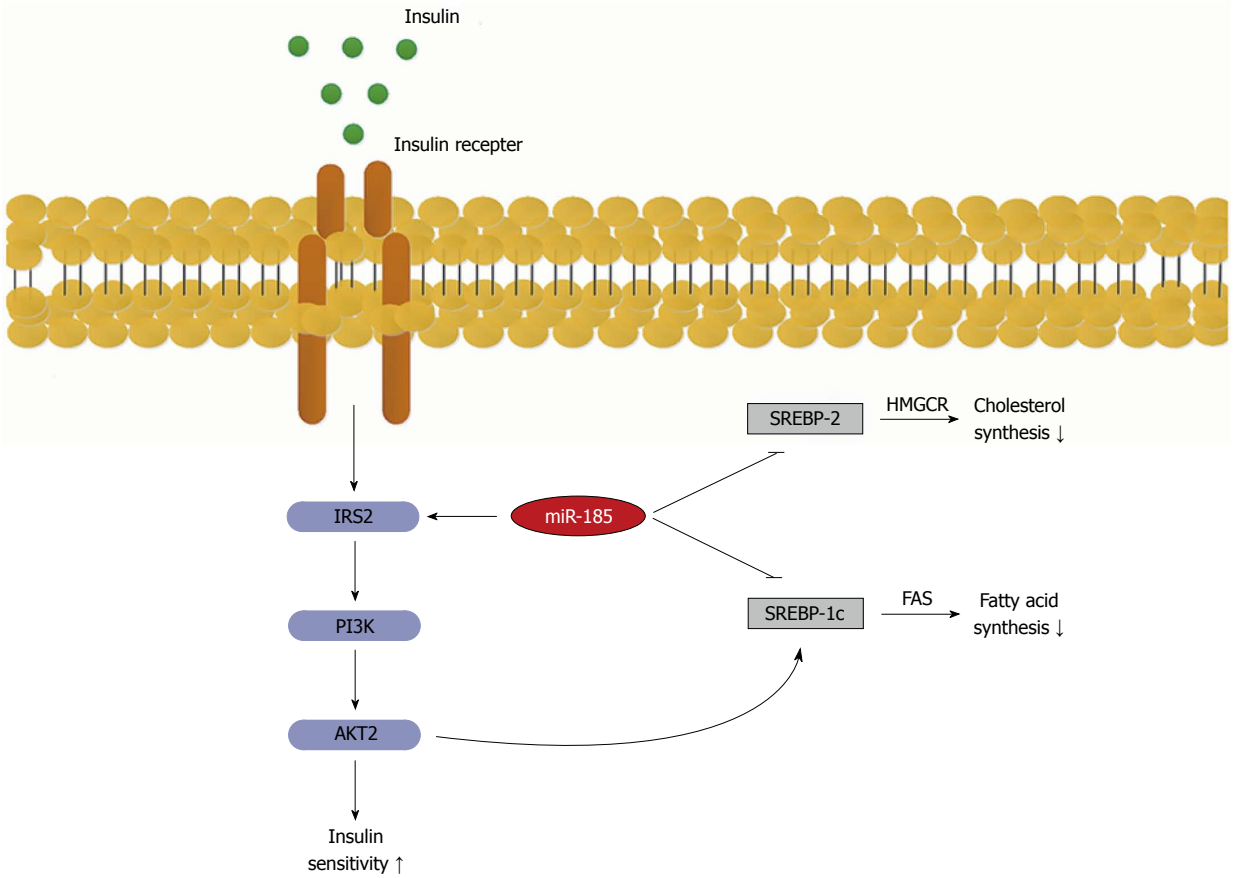

Figure 6 Role of microRNA-185 in the regulation of insulin signalling transduction and lipid metabolism. MicroRNA-185 up-regulates the key effector insulin receptor substrate-2 (IRS-2), which in turn activates the phosphatidylinositide 3-kinase/Akt2 signalling that increases insulin sensitivity. On the other hand, miR-185 represses SREBP-1c and 2, which ultimately results in decreased lipid synthesis. SREBP-1C: Sterol-regulatory element-binding proteins 1C; SREBP-2: Sterol-regulatory element-binding proteins 2; FAS: Fatty acid synthase.

of fatty acid and cholesterol homeostasis deserves attention[18-21]. Indeed, single miRNAs could regulate complex disease processes by targeting the biological pathways of multiple proteinases and transcription factors; increasing evidence supports key roles for miRNAs in regulating lipid metabolism and insulin sensitivity, *e.g.*, miR-33a and miR-33b target key enzymes involved in fatty acid oxidation[22]. In addition, miR-181d expression decreases triglyceride and cholesterol levels in cells[23]; miR-122 overexpression induces cholesterol and triglyceride biosynthesis in the adult liver[24]; and miR-126 affects insulin sensitivity in hepatocytes[25]. It has recently been reported that miR-185 regulates cholesterol metabolism by directly targeting the 3'-untranslated region (UTR) of hepatic scavenger receptor class B type I (SR-BI) and decreasing HDL uptake[26]. In our previous study using bioinformat-

ics, miR-185 was predicted to be involved in the regulation of both NAFLD pathogenesis and insulin resistance (Wang, Zhan *et al* unpublished data).

Herein, we uncovered a potential role of miR-185 in the regulation of hepatic lipid metabolism, the development of NAFLD, and insulin resistance. Wang *et al*[26] have recently shown that miR-185 was decreased in liver tissues from ApoE knockout mice after 8 wk on a HFD. In accordance with our results, miR-185 expression declined in a time-dependent manner in C57BL/6J mice fed a HFD from the 8th week, and miR-185 expression levels decreased in a time- and dose-dependent manner in response to PA in HepG2 cells (Figure 6).

These findings demonstrate that overexpression of miR-185 contributes to reduced fatty acid and cholesterol biosynthesis, which paralleled the observed decreases in mRNA levels of the fatty acid metabolism-related gene FAS and multiple cholesterol metabolism-related genes, including HMGCR, SREBP-2 and SREBP-1c. Inhibition of miR-185 in HepG2 cells caused elevated fatty acid and cholesterol biosynthesis, which was accompanied by increases in the mRNA levels of these key lipogenic genes. Similarly, Li *et al*[13] found that miR-185 inhibited fatty acid and cholesterol biosynthesis in prostate cancer cells, where it regulated SREBP-1 and SREBP-2 gene expression by directly binding their 3'-UTRs. In agreement with this, Yang *et al*[27] have demonstrated that miR-185 post-transcriptionally represses SREBP-2 expression. However, these authors found no significant alteration in HMGCR expression after treatment of HepG2 cells with miR185[27]. Such a discrepancy might result from differences in the experimental conditions between their study and ours.

SREBP-1 controls the genes involved in fatty acid biosynthesis, whereas SREBP-2 predominantly regulates cholesterol metabolism[28-31]. They often act as key regulators to induce the transcription of lipid-related genes, including FASN, FDFT1 and HMGCR, which results in an increase in fatty acid and cholesterol biosynthesis[32-35]. Our animal model provided strong evidence that miR-185 inhibits lipid metabolism. MiR-185 likely affects fatty acid and cholesterol metabolism in hepatic cells at least in part by inhibiting SREBP-1 and SREBP-2 mRNA expression.

Insulin resistance was markedly improved by miR-185. No previous studies have reported that miR-185 regulates the insulin-signalling pathway. Interestingly, Ryu *et al*[25] have demonstrated that miR-126 overexpression causes insulin resistance in hepatocytes through direct targeting of IRS-1 mRNA. Likewise, Karolina *et al*[36] found that miR-144 directly inhibits IRS1 at the mRNA and protein levels, and constitutes a critical component in insulin signalling. As shown above, miR-185 overexpression enhanced IRS-2 expression, and insulin stimulated its downstream kinases PI3K and Akt2 in hepatocytes. We found significantly improved insulin sensitivity after treatment of HFD animals with miR-185. Although miR-185 affected IRS-2 protein expression as shown above, it is unlikely that miR-185 directly regulates IRS-2, *i.e.*, by interaction with the 3'-UTR of the gene, since IRS-2 is devoid of seed sequences for miR-185 binding. One possibility is that miR-185 targets other genes that in turn regulate IRS-2; this hypothesis merits further exploration.

We comprehensively describe the regulatory effect of miR-185 on lipid metabolism and insulin signalling *in vivo* and *in vitro*. Overall, our findings define additional functional relevance of miR-185 that might be useful in the design of therapeutic strategies for treating NAFLD and improving insulin resistance.

COMMENTS

Background

Lipid metabolism and the insulin signalling pathway are frequently closely associated with non-alcoholic fatty liver disease (NAFLD). Therefore, identification of the regulators that control lipid metabolism and insulin resistance is critical.

Research frontiers

No previous studies have reported that an microRNA co-regulated lipid metabolism and the insulin signalling pathway.

Innovations and breakthroughs

This is the first study to demonstrate that microRNA-185 (miR-185) regulates lipid metabolism and insulin signalling *in vivo* and *in vitro*.

Applications

Our findings define additional functional relevance of miR-185 that might be useful in the design of therapeutic strategies for treating NAFLD and improving insulin resistance.

Terminology

Insulin signalling pathways includes the phosphatidylinositide 3-kinase/Akt pathway and Ras/MAPK pathway. MicroRNAs are endogenous non-protein coding small RNA molecules (22 nucleotides in length) that negatively regulate target gene expression by suppressing the translation of specific mRNAs.

Peer review

This is a well-written paper with well thought out experiments, and each finding in this study may be worth reporting. The paper reports the significance of miR185 in the regulation of lipid metabolism gene expression and blockade of the insulin signalling pathway *in vitro* and *in vivo*.

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RESEARCH REPORT

Proinflammatory effects and molecular mechanisms of interleukin-17 in intestinal epithelial cell line HT-29

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Abstract

AIM: To evaluate the proinflammatory effects and molecular mechanisms of interleukin (IL)-17 in intestinal epithelial cell line HT-29.

METHODS: HT-29 cells were cultured with IL-17, tumor necrosis factor (TNF)-α, or the combination of both IL-17 and TNF-α. Real-time PCR and Western blot were used to measure the gene expression levels of neutrophil chemokines CXCL1, CXCL2, CXCL5, CXCL6, IL-8 and TH-17 cell chemokine CCL20, the phosphorylation levels of p38 and TNF-α, and the expression level of IL-8, after using the p38 inhibitor in HT-29 cells. The stable Act1 knockdown HT-29 cell line was established to further test the phosphorylation changes of p38, after using IL-17 and TNF-α.

RESULTS: After HT-29 cells were cultured with IL-17 and TNF-α, the expression levels of neutrophil chemokines (CXCL1, CXCL2, CXCL5, CXCL6, IL-8) and Th17 chemokine (CCL20) significantly improved (24.96 ± 2.53, 28.47 ± 2.87, 38.08 ± 2.72, 33.47 ± 2.41, 31.7 ± 2.38, 44.37 ± 2.73, respectively), and the differences were all statistically significant ($P < 0.01$). Western blot results showed that IL-17 obviously enhanced the phosphorylation level of p38, which was induced by TNF-α. Compared with the control group, the expression level of IL-8 significantly declined (9.47 ± 1.36 *vs* 3.06 ± 0.67, $P < 0.01$) when TH-29 cells were cultured with IL-17 and TNF-α. p38 inhibition assay showed that the p38 pathway played an essential role in the inflammatory response induced by IL-17. p38 phosphorylation levels could not be changed after using IL-17 and TNF-α in the stable Act1 knockdown HT-29 cell line.

CONCLUSION: IL-17 significantly promoted the gene expression levels of TNF-α-induced neutrophil chemokines and Th17 cell chemokine. It is obvious that IL-17 and TNF-α have synergistic effects on p38.

Key words: IL-17; HT-29; TNF-α; Inflammatory bowel disease

Core tip: Our study revealed that interleukin (IL)-17 significantly promoted the gene expression levels of tumor necrosis factor (TNF)-α-induced neutrophil chemokines and Th17 cell chemokines. It is obvious that IL-17 and TNF-α have synergistic effects on p38.

Wang YL, Fang M, Wang XM, Liu WY, Zheng YJ, Wu XB, Tao R. Proinflammatory effects and molecular mechanisms of interleukin-17 in intestinal epithelial cell line HT-29. *World J Gastroenterol* 2014; 20(47): 17924-17931 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17924.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17924

INTRODUCTION

Ulcerative colitis and Crohn's disease are also known as inflammatory bowel disease (IBD)[1]. The disease refers to chronic inflammatory disorders of the gastrointestinal tract, which can easily recur. IBD occurs more often in males than females, and nowadays, the morbidity rate is showing an upward trend. IBD has become one of the most common digestive system diseases[2]. It is widely accepted that the interaction of genetic and environmental factors leads to the disease[3-5], but the pathogenesis is still unclear.

Hundorfean *et al*[6] found that the expression level of interlukin-17 (IL-17) significantly increased in the peripheral blood of patients with IBD, which implies that IL-17 may play an important role in the physiological and pathological processes of the disease. IL-17 and tumor necrosis factor (TNF) can accelerate inflammatory response by inducing various kinds of inflammatory cytokines in diseases such as IBD[7,8]; however, the molecular mechanism of the proinflammatory effects is still unknown. Wu *et al*[9] reported that IL-17 can induce neutrophil infiltration and related inflammatory cytokine expression, through the p38 pathway in myoblasts and fibroblasts; yet epithelial cell mechanisms were still rarely reported.

To deeply understand the pathogenesis of IBD, the molecular mechanisms of the proinflammatory effects of IL-17 and TNF-α in intestinal epithelial cell line HT-29 need to be studied. Since intestinal epithelial cell line HT-29 has normal colonic epithelial structures and functions, it has been the most common cell line used in laboratory to study the immunologic mechanisms of the intestinal mucosa[10,11]. Even though the IL-17 inhibitor has been proven to be ineffective in IBD treatments, blocking the other site of the pathway may prove to be hopeful in future studies. Therefore, it is of great importance to explore the mechanisms of action of IL-17.

MATERIALS AND METHODS

Materials

The following reagents were utilized for this study: McCoy's 5A medium (hyClone), recombinant human IL-17 and TNF-α (eBiosciences), Eastep Universal RNA Extraction Kit (Promega), Fist Strand cDNA Synthesis Kit (Promega), SYBR Premix Ex TaqTM (Takara), p38 inhibitor SB203580 (Sigma), BCA protein assay kit (Thermo), Phospho-p38 MAPK antibody (Beyotime), HRP-labeled donkey anti-goat IgG (Beyotime), mouse anti-human Act1 antibody (Biolegend), and goat anti-mouse IgG (Biolegend).

The following instruments were utilized for this study: CO_2 incubator (Thermo), PCR amplifier (Longgene), electrophoresis apparatus (Tanon), Gene Genius bioimaging system (BIO-RAD), polyacrylamide gel electrophoresis apparatus (Tanon), centrifugal machine (Debon), and microplate reader (Thermo).

Cell culture

Intestinal epithelial cell line HT-29 was cultured in McCoy's 5A medium, supplemented with 10% fetal bovine serum, 100 mg/mL of streptomycin and 100 U/mL of penicillin, in a 5% CO_2 humidified environment at 37 ℃. The cells were cultured to the exponential phase for use.

Reverse transcription-PCR

TH-29 cells were seeded in 12-well plates (5×10^5 cells/well), the fetal bovine serum was reduced to 0.5% as they reached about 70% confluence, and the plates were cultured overnight. Then, rhIL-17 (50 ng/mL), rhTNF-α (0.5 ng/mL), or the combination of rhIL-17 and rhTNF-α was added into the plates. The cells were cultured for another 6 h, the samples were collected, and TRIzol (1 mL TRIzol per 5-10 × 10^6 cells) was added to lyse the cells. Before adding TRIzol, cells were not washed with a buffer solution, since the cellular RNA can be easily degraded. Total RNA was isolated using an Eastep Universal RNA Extraction kit and reverse transcribed with a Fist Strand cDNA Synthesis kit, according to the manufacturer's instructions. Finally, real-time fluorescence quantitative PCR (SYBR Green I) was performed using 200 ng of cDNA. The reaction system was instructed by the SYBR Premix Ex Taq™ specifications purchased from Takara. The reaction conditions were as follows: initial denaturation at 95 ℃ for 10 min, followed by 40 cycles of denaturation at 95 ℃ for 15 s and annealing at 60 ℃ for 30 s. The fluorescence signal was then collected. After amplification, the melting curve from 65 ℃ to 95 ℃ was analyzed to exclude non-specific PCR products and amplified primer dimers. Agarose gel electrophoresis was then used to analyze the specificity of the product. GAPDH was amplified as a control for the amount of cDNA in each sample, and the expression level of each gene was computed. The gene levels of neutrophil chemokines and TH-17 cell chemokine (*CXCL1*, *CXCL2*, *CXCL5*, *CXCL6*, *IL-8* and *CCL20*) were examined. All primers used in our study were designed according to the sequences published online (GenBank) and synthesized by Invitrogen (Table 1).

Cells were cultured as described above, and rhIL-17, rhTNF-α, and p38 inhibitor SB203580 were added into the plate of the experiment groups. The same procedure was conducted to detect the gene expression level of IL-8.

Western blot

TH-29 cells were seeded in 6-well plates (8×10^5 cells/well), fetal bovine serum was reduced to 0.5% as they

Table 1 Primers used in RT-PCR

Gene	Forward	Reverse
CXCL1	AGATTCTATGTTAATATTTTAGGTGTAAAATAAT	AACTAACTTGGGGTTGACATTTC
CXCL2	CTTCTATTTATTTATTTATTTATTTATTTGTTTGTTTT	GAACTAACTTGGGTTTGACCTAAA
CXCL5	TGGCCCCTTTCACAGAGTAG	CTAAAAACCCGACAGGCATC
CXCL6	AGTTTACAGCTCAGCTAATGAAGTACTAAT	CGGTAAGACTTTAAGGAATGTATGATA
IL-8	GAATTGAATGGGTTTGCTAGA	CACTGTGAGGTAAGATGGTGG
CCL20	CTGGCTGCTTTGATGTCAGT	CGTGTGAAGCCCACAATAAA

IL-8: Interleukin-8.

reached about 70% confluence, and the cells were cultured overnight. Then, rhIL-17 (50 ng/mL), rhTNF α (0.5 ng/mL), or the combination of rhIL-17 and rhTNF α was added into the plates. The samples were collected 10, 15 and 30 min later. At each time point, the medium was removed and the cells were washed with PBS solution; M2 was then added and the plate was left on ice for 30 min to lyse the cells. The cells were collected into a centrifuge tube with a cell scraper and lysed with ultrasonic irradiation for 3 s, 2 times. Cell debris was removed by lysate centrifugation at 12000 g for 2 min at 4 ℃ and protein concentration was determined using the BCA Protein assay kit. The working liquid was prepared according to the manufacturer's instructions. The standard curve was made, the plate was incubated at 37 ℃ for 30 min, and the optical density (570) was tested with a microplate reader. Finally, protein concentration was computed according to the standard curve. All specimens were adjusted to have the same concentration. After mixing the protein supernate with the buffer, the liquid was boiled for 10 min, cooled, centrifuged, and resolved by 12% SDS-PAGE. The protein was then transferred to a nitrocellulose membrane. An enhanced chemiluminescence (ECL) based Western blot was performed using a primary anti-p38 antibody, followed by incubation with a horseradish peroxidase-labeled secondary antibody for chemiluminescence detection. Briefly, the 5% bovine serum albumin (BSA) blocked membrane was incubated for 12 h at 4 ℃ with the primary antibody (1:1000). Then, the membrane was washed and incubated with the horseradish peroxidase conjugated rabbit anti-mouse IgG for 60 min at room temperature. After washing, the protein was tested with an ECL Western blot analysis detection system.

Establishing the Act1 silenced cell line

HT-29 cells were cultured in 96-well plates. When the cells reached 80% confluence, Metafectene transfection reagents were used to transfer HT-29 targeted small interfering RNA into the HT-29 cells. The plasmid with the best knockdown performance (tested through transient transfection) was selected to establish a stable cell line, and the cells were cultured for subsequent experiments. Immunocytochemistry was performed to identify the transfected cells. The cells were briefly blocked with calf serum (diluted with PBS solution) for 15 min, the serum was removed, and the cells were incubated with a mouse anti-human Act1 antibody for 12 h at 4 ℃. Unbounded antibodies were washed off with PBS solution, and cells were again incubated with a goat anti-mouse IgG for one hour at room temperature, followed by mounting and microscopic examination.

The transfected cells were seeded in 6-well plates (8×10^5 cells/well), fetal bovine serum was reduced to 0.5% as they reached about 80% confluence, and the cells were cultured overnight. Then, rhIL-17 (50 ng/mL), rhTNF-α (0.5 ng/mL) or the combination of rhIL-17 and rhTNF-α was added into the plates. The samples were collected 15 min later. The same method described above was used to test the expression levels of p-p38 and p-ERK.

Statistical analysis

SPSS 17.0 software was used for statistical analyses in this study. All data are expressed as mean ± SD, and *t*-test was applied in performing the statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Neutrophil and TH-17 chemokine expression in HT-29 cells

Figure 1 showed that the chemokine RNA expression levels (A-E: neutrophil chemokines; F: TH-17 chemokine) were comparatively low, when IL-17 and TNF-α were used separately. However, when the chemokines were used together, the expression of mRNAs coding for all these mediators became strongly upregulated. The differences were statistically significant ($P < 0.01$).

p38 and ERK phosphorylation levels

When IL-17 was used alone, no obvious phosphorylation occurred on p38 and ERK; and when TNF-α was used alone, the phosphorylation that occurred on p38 and ERK was very low. In contrast, when IL-17 and TNF-α were combined together, obvious phosphorylation can be easily observed; the longer IL-17 and TNF-α were combined together, the stronger the phosphorylation became (Figure 2).

Expression level of IL-8 in the presence of p38 inhibitor

Whether or not the p38 inhibitor was added, the IL-17 and TNF-α combination could still significantly improve the expression level of IL-8; and compared with cells that were cultured without the p38 inhibitor, the expres-

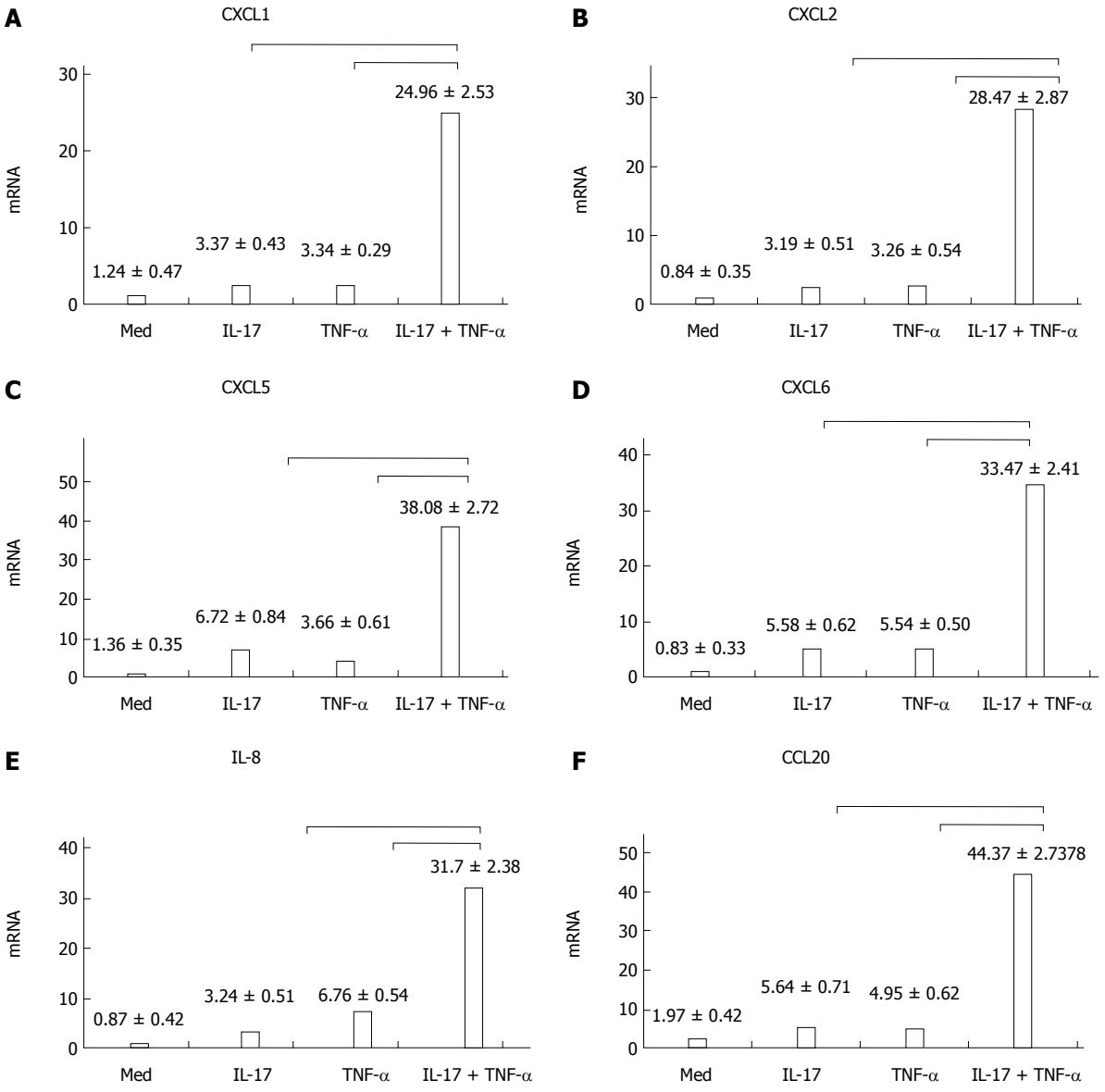

Figure 1 Chemokine expression when HT-29 cells were cultured with interleukin-17 and/or tumor necrosis factor-α. When interleukin (IL)-17 and tumor necrosis factor (TNF)-α were used separately, the expression levels of *CXCL1*, *CXCL2*, *CXCL5*, *CXCL6*, *IL-8* and *CCL20* in HT-29 cells were comparatively low. However, when IL-17 and TNF-α were used together, the expression levels became strongly upregulated; A: IL-17 + TNF-α *vs* IL-17, $t = 30.424$, $P = 0.000$; IL-17 + TNF-α *vs* TNF-α, $t = 30.438$, $P = 0.000$; B: IL-17 + TNF-α *vs* IL-17, $t = 35.273$, $P = 0.000$; IL-17 + TNF-α *vs* TNF-α, $t = 35.125$, $P = 0.000$; C: IL-17 + TNF-α *vs* IL-17, $t = 85.718$, $P = 0.000$; IL-17 + TNF-α *vs* TNF-α, $t = 94.199$, $P = 0.000$; D: IL-17 + TNF-α *vs* IL-17, $t = 50.165$, $P = 0.000$; IL-17 + TNF-α *vs* TNF-α, $t = 50.224$, $P = 0.000$; E: IL-17 + TNF-α *vs* IL-17, $t = 54.585$, $P = 0.000$; IL-17 + TNF-α *vs* TNF-α, $t = 44.036$, $P = 0.000$; F: IL-17 + TNF-α *vs* IL-17, $t = 56.272$, $P = 0.000$; IL-17 + TNF-α *vs* TNF-α, $t = 57.410$, $P = 0.000$. IL-8: Interleukin-8.

sion level of IL-8 significantly reduced with the advent of the p38 inhibitor, in all the three groups (Figure 3).

Phosphorylation level of IL-8 in Act1 silenced HT-29 cell line

Immunocytochemistry was performed to identify the Act1 silenced cell. Fluorescent intensity greatly decreased as compared with the control group (Figure 4) and the positive rate reached up to 90%. In the control group, the IL-17 and TNF-α combination greatly elevated the phosphorylation level of p38; while in the Act1 silenced treatment group, no obvious change occurred (Figure 5).

DISCUSSION

To deeply understand the pathogenesis of IBD, the molecular mechanisms of the proinflammatory effects of IL-17 and TNF-α in the intestinal epithelial cell line HT-29 were investigated in this study. We analyzed the expression levels of neutrophil and TH-17 cell chemokines, and the phosphorylation levels of p38 in HT-29 cells - when cultured with IL-17 and/or TNF-α. The results further confirmed that IL-17 could obviously enhance TNF-α induced p38 phosphorylation, and that the p38 pathway played an essential role in IL-17 induced inflammatory response.

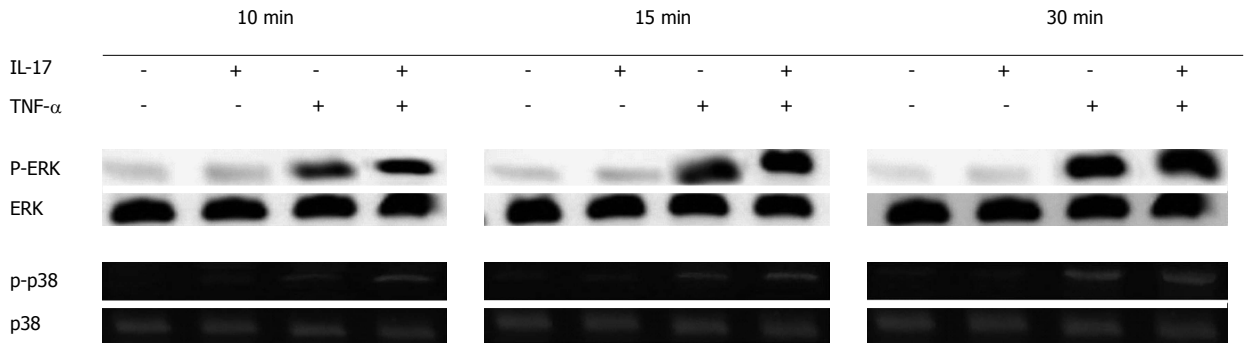

Figure 2 p38 and extracellular signal-regulated kinase phosphorylation levels. When interleukin (IL)-17 and tumor necrosis factor (TNF)-α were used separately, the phosphorylation levels of p38 and extracellular signal-regulated kinase (ERK) were very low. However, when IL-17 and TNF-α were combined together, the phosphorylation levels of p38 and ERK improved significantly, and the longer IL-17 and TNF-α were combined together, the stronger the phosphorylation became.

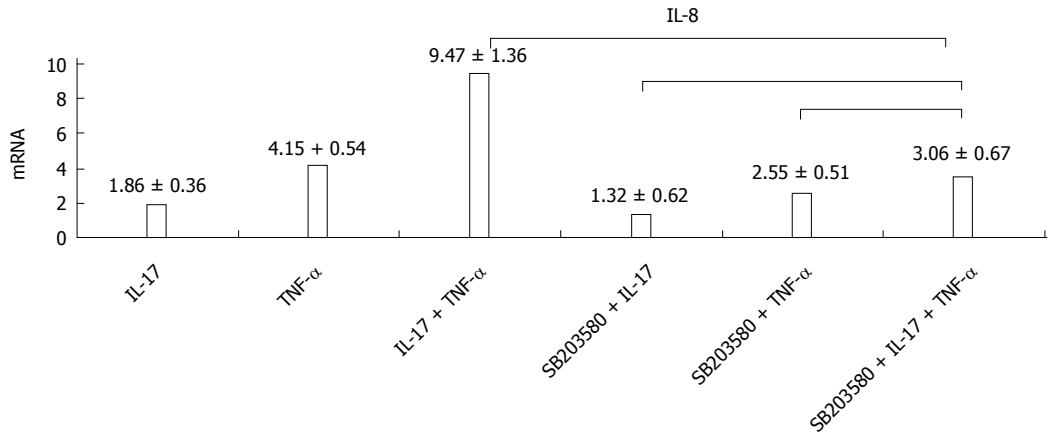

Figure 3 p38 inhibitor decreases the expression of interleukin-8. When p38 inhibitor was used, the interleukin (IL)-8 expression levels declined in all three groups. IL-17 + TNF-α *vs* S + IL-17 + TNF-α, $t = 103.701$, $P = 0.000$; S + IL-17 + TNF-α *vs* S + TNF-α, $t = -4.131$, $P = 0.07$; S + IL-17 + TNF-α *vs* S + IL-17, $t = -11.509$, $P = 0.000$. IL-8: Interleukin-8; TNF-α: Tumor necrosis factor-α; S: SB203580.

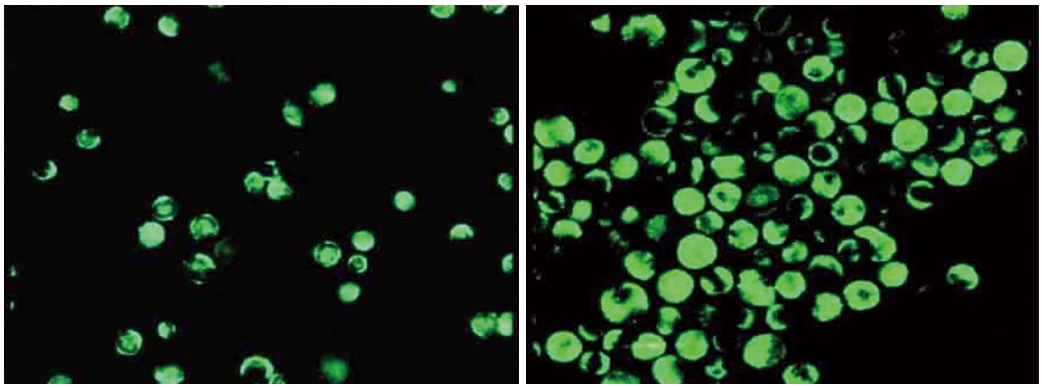

Figure 4 Act1 silenced HT-29 cells. Compared with the control group (A), the expression level of Act1 was comparatively low in Act1 silenced cells (B).

Frontier of IL-17

Intestinal epithelium is the physical barrier between the intestinal tract and the external environment, playing an important role in the intestinal immune system. The cell line HT-29 used in our research is a human colonic epithelial cell line - the most common cell line used in laboratory to study the immunologic mechanisms of the intestinal mucosa. As the most representative member of the IL-17 family, IL-17 received much attention in recent years. Various studies suggested that multiple chemokines can be induced by IL-17 in epithelial cells to promote neutrophil infiltration in the lesion area[12,13]. It is currently known that IL-17 is involved in the pathological process of autoimmune diseases[14,15]. IL-17 monoclonal antibodies can be used for treating psoriasis, rheumatoid arthritis and uveitis, which are now in the clinical trial phase[16]. On the contrary, when the monoclonal antibodies were used in IBD treatment, the illness exacerbated[17,18]. This phe-

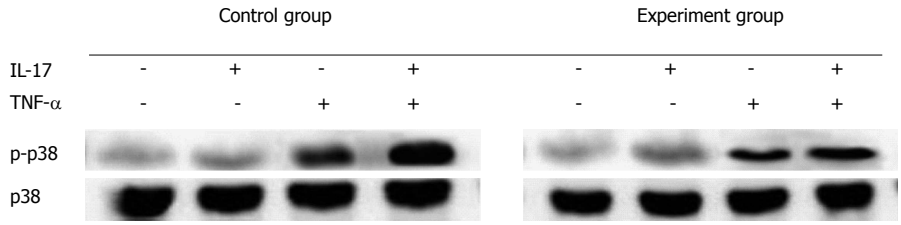

Figure 5 Phosphorylation levels of p38 in Act1 silenced HT-29 cell line. In the control group, interleukin (IL)-17 combined with tumor necrosis factor (TNF)-α could greatly improve the phosphorylation level of p38. In Act1 silenced cells, the phosphorylation level of p38 remained unchanged, when IL-17 and TNF-α were used together.

nomenon suggested that IL-17 may have a special mode of action in IBD. TNF-α is another cytokine that plays an important role in the immune regulation of IBD[19]. Hence, in our study, we combined IL-17 with TNF-α, to investigate whether IL-17 could act alone or together with TNF-α in HT-29 cells.

IL-17 combined with TNF-α upregulates HT-29 chemokine expression levels

Chemokine factors CXCL1, CXCL2, CXCL5, CXCL6 and IL-8 play important roles in recruiting neutrophils in inflammation reactions; therefore, we detected their expression levels to monitor the proinflammatory effects of IL-17. We discovered that when IL-17 or TNF-α was used alone for cell stimulation, expression levels of chemokine factors were comparatively low, but when IL-17 and TNF-α were combined, the expression levels elevated significantly. The results suggest that there may be a synergy between IL-17 and TNF-α. The inflammatory response caused by IL-17 was achieved by the MAPK signal pathway, which mediates the stability of mRNAs[12,20]. TNF-α is a genetic transcription starter that can promote gene transcription[21,22]. The mRNAs regulated by IL-17 possess a similar sequence. There is an AU-rich sequence in the 3' putative untranslated regions of the mRNA, which can be grasped by zinc finger proteins; and thus, the mRNA can be recognized and degraded by exosome complex[23]. For relatively stable mRNAs, the lack of the AU-rich sequence does not make it mediated by IL-17. Moreover, IL-17 can elevate the chemokine factor CCL20 through TNF-α, and CCL20 can combine with CCR6 on the cell surface to further recruit TH-17 cells, causing more IL-17 to be expressed. This would create a positive feedback and further promote inflammatory reactions[24,25]. Therefore, we can assume that IL-17 may improve the expression levels of related cytokines by stabilizing their mRNAs through the MAPK pathway together with TNF-α.

The proinflammatory mechanism of IL-17

In our study, p38 inhibitor could decrease IL-8 expression levels in HT-29 cells. This result suggests that the two cytokines may accelerate inflammatory response through the p38 pathway, which is similar to Guo's report[26]. We also found that when IL-17 and TNF-α were used together to stimulate the Act1 silenced cells, p38 phosphorylation level significantly decreased, compared with the control group. The result further confirmed that the Act1-dependent p38 pathway is a very important factor for IL-17-mediated proinflammatory responses. Act1 is an important receptor protein in the IL-17 mediated signal pathway and its expression level affects the activation of the MARK pathway by IL-17[12,27]. Kang *et al*[28] discovered that inflammatory response could be reduced by lowering the expression level of Act1. It is well known that IL-17 can't cause inflammatory response alone. It is commonly believed that several factors working together triggered the inflammatory response[29,30].

In conclusion, we discover that IL-17 can synergistically promote gene expression of TNF-α-mediated neutrophil chemokines and TH-17 cell chemokines. IL-17 obviously enhances the TNF-α-induced p38 phosphorylation, and the p38 pathway plays an essential role in IL-17-induced inflammatory response. In our study, we only examine the Act1-dependent p38 pathway, but it is still unclear whether other pathways (ERK, PI3K-AKT) or cytokines also participate in the function of IL-17; thus, further studies need to be performed.

COMMENTS

Background

Ulcerative colitis and Crohn's disease are known as inflammatory bowel disease (IBD). It refers to chronic inflammatory disorders of the gastrointestinal tract, which can easily recur. IBD occurs more often in males than females, and nowadays, the morbidity rate is showing an upward trend. IBD has become one of the most common digestive system diseases. It is widely accepted that the interaction of genetic and environment factors leads to the disease, but the pathogenesis is still unclear.

Research frontiers

Previous studies show that interlukin-17 (IL-17) expression levels significantly increased in the peripheral blood of patients with IBD, which implies that IL-17 and tumor necrosis factor (TNF) can accelerate the inflammatory response through inducing various kinds of inflammatory cytokines in diseases such as IBD; however, the molecular mechanisms of the proinflammatory effects are still unknown. IL-17 can induce neutrophil infiltration and related inflammatory cytokine expression through the p38 pathway in myoblasts and fibroblasts, yet epithelial cell mechanisms were still rarely reported.

Innovations and breakthroughs

The authors discovered that IL-17 can synergistically promote gene expression of TNF-α-mediated neutrophil chemokines and TH-17 cell chemokine. IL-17 obviously enhanced TNF-α-induced p38 phosphorylation and the p38 pathway plays an essential role in IL-17-induced inflammatory response.

Applications

This study is helpful in deeply understanding the pathogenesis of IBD, and the molecular mechanisms of the proinflammatory effects of IL-17 and TNF-α on intestinal epithelial cell line HT-29. Even though the IL-17 inhibitor proved to be ineffective in IBD treatments, blocking the other site of the pathway may prove

to be hopeful in future studies.

Peer review

This is a very interesting manuscript about the proinflammatory effects and molecular mechanisms of IL-17 in the intestinal epithelial cell line HT-29.

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Lean-non-alcoholic fatty liver disease increases risk for metabolic disorders in a normal weight Chinese population

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Abstract

AIM: To study the prevalence and clinical biochemical, blood cell and metabolic features of lean-non-alcoholic fatty liver disease (lean-NAFLD) and its association with other diseases.

METHODS: Demographic, biochemical and blood examinations were conducted in all the subjects in this study. We classified the subjects into four groups according to their weight and NAFLD status: lean-control, lean-NAFLD [body mass index (BMI) < 24 kg/m^2], overweight-obese control and overweight-obese NAFLD. One-way analysis of variance (ANOVA) was used to compare the means of continuous variables (age, BMI, blood pressure, glucose, lipid, insulin, liver enzymes and blood cell counts) and the χ^2 test was used to compare the differences in frequency of categorical variables (sex, education, physical activity, smoking, alcohol consumption and prevalence of hypertension, hyperlipidemia, diabetes, metabolic syndrome central obesity and obesity). Both univariate and multivariate logistic regression models were adopted to calculate odds ratios (ORs) and predict hyperlipidemia, hypertension, diabetes and metabolic syndrome when we respectively set all controls, lean-control and overweight-obese-control as references. In multivariate logistic regression models, we adjusted potential confounding factors, including age, sex, smoking, alcohol consumption and physical activity.

RESULTS: The prevalence of NAFLD was very high in China. NAFLD patients were older, had a higher BMI, waist circumference, blood pressure, fasting blood glucose, insulin, blood lipid, liver enzymes and uric acid than the controls. Although lean-NAFLD patients had lower BMI and waist circumstance, they had significantly higher visceral adiposity index than overweight-obese controls. Lean-NAFLD patients had comparable triglyceride, cholesterin and low-density lipoprotein cholesterin to overweight-obese NAFLD patients. In blood cell examination, both lean and overweight-obese NAFLD was companied by higher white blood cell count, red blood cell count, hemoglobin and hematocrit value. All NAFLD patients were at risk of hyperlipidemia, hypertension, diabetes and metabolic syndrome (MetS). Lean-NAFLD was more strongly associated with diabetes (OR = 2.47, 95%CI: 1.14-5.35), hypertension (OR = 1.72, 95%CI: 1.00-2.96) and MetS (OR = 3.19, 95%CI: 1.17-4.05) than overweight-obese-NAFLD (only OR for MetS was meaningful: OR = 1.89, 95%CI: 1.29-2.77). NAFLD patients were more likely to have central obesity (OR = 1.97, 95%CI: 1.38-2.80), especially in lean groups (OR = 2.17, 95%CI: 1.17-4.05).

CONCLUSION: Lean-NAFLD has unique results in de-

mographic, biochemical and blood examinations, and adds significant risk for diabetes, hypertension and MetS in lean individuals.

Key words: Lean-non-alcoholic fatty liver disease; Metabolic disorder; Diabetes; Risk; Chinese

Core tip: Obesity is an important risk factor for non-alcoholic fatty liver disease (NAFLD). NAFLD can also occur in lean subjects. Chinese people have lower body mass index than Americans and Europeans, but a similar prevalence of NAFLD. There might be different metabolic characters in Chinese population. We conducted this study to characterize metabolic features of lean-NAFLD and identify its association with metabolic disorders.

Feng RN, Du SS, Wang C, Li YC, Liu LY, Guo FC, Sun CH. Lean-non-alcoholic fatty liver disease increases risk for metabolic disorders in a normal weight Chinese population. *World J Gastroenterol* 2014; 20(47): 17932-17940 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17932.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17932

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become an important public health issue because of its high prevalence[1]. It has been estimated that its prevalence varies between 20% and 30% in developed countries and Middle East[2]. Japan, China and India subcontinent have a similar prevalence (20%-30% in Japan, 15%-30% in China and 16%-32% in the Indian subcontinent)[3]. NAFLD is a hepatic manifestation of metabolic syndrome (MetS) and its long-term prognoses include non-alcoholic steatohepatitis (NASH), liver cirrhosis, even hepatocellular carcinoma (HCC)[1]. Patients with NAFLD are more likely to have insulin resistance (IR), abnormal glucose metabolism and higher risk for the development of diabetes[4]. It is an independent risk factor for chronic diseases, such as cardiovascular and renal diseases[5,6], and it is also associated with colorectal disease, atrial fibrillation and hypothyroidism[7-9].

Obesity is strongly associated with many metabolic diseases[10], including NAFLD, and bariatric surgery is recommended as a promising treatment[11]. However, NAFLD can also be observed in non-obese individuals and has its own metabolic characteristics, such as higher transaminase and insulin levels, less insulin sensitivity than non-obese controls; lower fasting glucose, less advanced necro-inflammatory activity and fibrosis compared with obese-NAFLD[12]. The prevalence of NAFLD in non-diabetic, non-obese adults was 23.4% (16.1% in the normal-weight group and 34.4% in the overweight group) in the study of Kim *et al*[13]. The prevalence varied from 15% to 21% in non-obese Asians [body mass index (BMI) < 25][14]. NAFLD can be considered as an early predictor of metabolic disorders and a major cause of cryptogenic liver disease in normal-weight population[12,13]. Chinese people have lower BMI, but have a similar prevalence of NAFLD with Western people[13,15]. We suspected that lean-NAFLD is more serious in China and has different clinical characteristics.

The lack of knowledge of the prevalence and characteristics of lean-NAFLD in the Chinese population prompted us to conduct this study to: (1) define the prevalence and characterize the clinical biochemical, blood cell and metabolic features of lean-NAFLD; and (2) clarify the association between lean-NAFLD and chronic diseases, including diabetes, hypertension, hyperlipidemia and MetS.

MATERIALS AND METHODS

Ethics

The Ethics Committee of Harbin Medical University approved this study. Written Informed Consent was obtained from all participants.

Subjects

We randomly selected 2000 subjects who received annual physical examinations at Physical Examination Center of the Second Affiliated Hospital of Harbin Medical University from February 2012 to May 2013. The exclusion criteria were as follows: previous/current excessive alcohol intake (male > 20 g/d; female > 10 g/d), hepatitis, malignancies, pregnancy, long-term use of estrogens, tamoxifen, or corticosteroids, and absence of any of the anthropometric measurement, or laboratory analysis. Finally, 1779 adults aged 20-70 years were included.

Measurements

Each of these subjects was interviewed privately by trained interviewers, to complete a questionnaire that included questions regarding name, age, gender, education level, history of disease, drug or tobacco use, physical activity status and alcohol consumption. Smoking was defined as never, ⩽ 1 cigarettes/d, ⩽ 10 cigarettes/d, ⩽ 20 cigarettes/d, and > 20 cigarettes/d; physical activity intensity was categorized into three groups: none, without any regular hard physical activity; moderate, having hard physical activity at least once a week regularly; and vigorous, having hard physical activity (leisure time or occupational) at least three times a wk. Alcohol consumption was calculated by the amount of alcohol drinks multiplied by the frequency.

Current weight, height and fat mass (FM) were measured using the electric impedance method with a body fat mass analyzer (ioi 353; Janex Medical, Seoul, Korea), with the examinees minimally clothed and wearing no socks. Weight and FM were recorded to the nearest 0.1 kg and height was measured in a standing position to the nearest 0.1 cm. BMI was calculated as weight in kilograms divided

by height in meters squared. Waist circumference (WC) was measured at the umbilical level, using un-stretchable tape meter, without any pressure to body surface, and was recorded to the nearest 0.1 cm. A well-trained examiner measured all anthropometric indices. A qualified physician measured their blood pressure twice, and there was at least a 30-s interval between these two separate measurements, and thereafter the mean of the two measurements was recorded as definitive blood pressure.

Laboratory analysis

Blood samples were taken after > 10 h overnight fasting. A complete blood count was measured using an automated laser-based hematology analyzer (Sysmex XE-2100, Kobe, Japan). Hemoglobin (HGB), red blood cell count (RBC), hematocrit value (HCT), white blood cell count (WBC) and its subtypes-neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), basophils (BASO) were all recorded. The biochemical indicators detected included fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP), serum creatinine (CRE), blood urea nitrogen (BUN) and serum uric acid (UA). All of these variables were determined using a ROCHE Modular P800 Automatic Biochemical Analyzer (Roche Diagnostics, Mannheim, Germany). A ROCHE Elecsys 2010 Chemiluminescence Immune Analyzer (Roche Diagnostics) measured the serum fasting insulin concentration. The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated as previously described[16]. Visceral adiposity index was calculated according to a published formula: male: visceral adiposity index (VAI) = WC/[39.68 + (1.88 × BMI)] × (TG/1.03) × (1.31/HDL); female: VAI = WC/[36.58 + (1.89 × BMI)] × (TG/0.81) × (1.52/HDL) (WC in cm, both TG and HDL in mmol/L)[17].

Diagnostic criteria

An abdominal ultrasonographic examination was performed to determine liver fatty infiltration using a 3.5-MHz probe (SSI-8000, Philips, Netherlands) by an experienced ultrasonographist, who was blind to the subjects' disease history or blood laboratory analysis. The liver of each participant was assessed for size, contour, echogenicity, structure and posterior beam attenuation.

According to the guidelines for NAFLD management formulated by the Chinese National Workshop on Fatty Liver Disease in 2010[18], NAFLD can be diagnosed according to the following criteria: (1) alcohol consumption < 140 g/wk for male adults and < 70 g/wk for female adults; (2) absence of viral hepatitis [hepatitis B virus (HBV)/hepatitis C virus (HCV)], hepatolenticular degeneration, autoimmune diseases, a history of total parenteral nutrition, or intake of any hepatotoxic drugs (*e.g.*, tamoxifen, amiodarone, sodium valproate, methotrexate, and glucocorticoid); and (3) ultrasonographic examination suggesting fatty infiltration in liver.

MetS was defined according to the International Diabetes Federation criteria[19] as waist circumference (WC) ⩾ 90 cm for men and WC ⩾ 80 cm for women plus at least two of the following components: (1) hypertriglyceridemia: TG ⩾ 1.7 mmol/L (150 mg/dL), or under specific treatment for this lipid abnormality; (2) low HDL-C: HDL-C < 1.03 mmol/L (40 mg/dL) for men and < 1.29 mmol/L (50 mg/dL) for women or under specific treatment for this lipid abnormality; (3) raised blood pressure: systolic blood pressure (SBP) ⩾ 130 mmHg, or diastolic blood pressure (DBP) ⩾ 85 mmHg, or having previously diagnosed hypertension; and (4) hyperglycemia: fasting glucose ⩾ 5.6 mmol/L (110 mg/dL).

A patient was defined as having hypertension if his blood pressure was higher than 140/90 mmHg or with disease history, and diabetes mellitus was defined when fasting blood glucose was higher than 7.0 mmol/L or with disease history. If serum triglycerides was ⩾ 1.70 mmol/L or serum cholesterol ⩾ 5.18 mmol/L or with disease history, hyperlipidemia would be diagnosed.

Statistical analysis

The subjects were divided into four groups according to their weight and NAFLD status: Lean control and NAFLD, overweight-obese control and NAFLD (lean: BMI < 24 kg/m^2, overweight-obese: BMI ⩾ 24 kg/m^2). Statistical analysis was performed using SPSS (version 16.0; Beijing Stats Data Mining Co. Ltd, Beijing, China). The χ^2 test was used to test variation in frequency, and analysis of variance (ANOVA) was used to assess differences in the means of continuous variables. Data are presented as means ± SD. Multiple variable logistic regression was used to calculate the odds ratios (ORs) of diabetes, hypertension, hyperlipidemia and MetS after adjusted for potentially confounding variables, including age, sex, BMI, smoking, alcohol consumption and physical activities. All *P* values were two-tailed, and *P* value < 0.05 was considered statistically significant.

RESULTS

Demographic characteristics

The basic demographic characteristics (age, gender composition, education, physical activity, smoking, alcohol consumption and chronic diseases) of the 1779 participants are summarized in Table 1. NAFLD had a male predominance, and BMI and blood pressure were higher in NAFLD patients than in controls in the same group. Overweight-obese-NAFLD patients had higher body fat, VAI and blood pressure than the lean-NAFLD group. Diabetes, hypertension, hyperlipidemia and MetS were more common in lean and overweight-obese NAFLD patients than in healthy controls.

Comparison of biochemical indicators

All biochemical indicators are shown in Table 2, includ-

Table 1 Baseline demographic characteristics of non-alcoholic fatty liver disease patients and controls

	Lean-NAFLD (n = 731)		Overweight-obese-NAFLD (n = 1048)		P value
	Controls	NAFLD	Controls	NAFLD	
Participants, n (%)	597 (81.67)	134 (18.33)	284 (27.10)	764 (72.90)	
Age (yr)	43.19 ± 11.59	48.17 ± 10.5[a]	46.72 ± 11.15[b]	46.92 ± 11.19	< 0.01
Male (%)	28.48	54.48	51.41	72.25	< 0.01
Education (%)					
Never	0.66	1.92	0.90	0.87	0.30
Primary	0.66	0.00	2.26	1.56	
Junior	4.20	2.88	6.33	3.65	
Senior	15.27	11.54	19.46	18.92	
College	63.50	66.35	58.37	61.98	
Postgraduate	15.71	17.31	12.67	13.02	
BMI (kg/m^2)	21.37 ± 1.71	22.74 ± 1.13[a]	25.98 ± 1.66[b]	27.57 ± 2.63[a,c]	< 0.01
Body weight (kg)	57.64 ± 7.14	64.09 ± 7.31[a]	71.32 ± 9.49[b]	78.81 ± 11.64[a,c]	< 0.01
Body fat (%)	24.83 ± 5.40	26.17 ± 4.96[a]	29.61 ± 4.73[b]	30.05 ± 4.49[c]	< 0.01
Body fat mass (kg)	14.26 ± 3.37	16.59 ± 2.68[a]	20.91 ± 3.34[b]	23.59 ± 4.61[a,c]	< 0.01
WC (cm)	75.50 ± 7.06	82.42 ± 6.29[a]	87.06 ± 7.16[b]	93.45 ± 8.70[c]	< 0.01
VAI	1.52 ± 1.99	2.04 ± 2.21[a]	1.85 ± 2.32[b]	2.08 ± 1.50	< 0.01
SBP (mmHg)	119.48 ± 15.45	126.46 ± 16.24[a]	129.37 ± 19.86[b]	132.20 ± 17.29[c]	< 0.01
DBP (mmHg)	74.04 ± 10.04	80.05 ± 11.74[a]	79.33 ± 12.17[b]	82.84 ± 10.57[a,c]	< 0.01
Physical activity (%)					
None	83.92	89.52	80.27	85.10	0.45
Moderate	14.98	10.48	18.39	13.86	
Vigorous	1.10	0.00	1.35	1.04	
Drinking (%)	23.82	36.89	39.25	49.02	< 0.01
Smoking (%)					
Never	86.09	82.52	81.45	68.34	< 0.01
≤ 1 cigarettes/d	1.10	0.97	1.36	2.25	
≤ 10 cigarettes/d	5.08	3.88	5.43	7.96	
≤ 20 cigarettes/d	5.74	8.74	7.24	14.01	
> 20 cigarettes/d	1.99	3.88	4.52	7.44	
Diabetes (%)	4.58	15.67	9.54	16.49	< 0.01
Hypertension (%)	12.23	34.71	32.08	43.67	< 0.01
Hyperlipidemia (%)	34.20	49.25	47.69	59.47	< 0.01
MS (%)	3.06	14.53	26.47	46.64	< 0.01
Central obesity (%)	14.96	29.41	69.55	84.64	< 0.01
Obesity (%)	0.00	0.00	11.97	37.70	< 0.01

[a]P < 0.05 *vs* control in same weight group; [b]P < 0.05 *vs* Lean-control group, [c]P < 0.05 *vs* Lean-NAFLD group. NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; WC: Waist circumstance; VAI: Visceral adiposity index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MS: Metabolic syndrome.

ing FBG, fasting insulin, TC, TG, HDL-C, LDL-C, ALT, AST, ALP, GGT, UA, BUN and CREA. NAFLD patients tended to have higher FBG, fasting insulin, HOMA-IR, TG, LDL-C, ALT, AST, ALP, GGT and UA, and lower HDL-C compared with healthy controls. Fasting insulin, HOMA-IR TC, AST, GGT, UA and CREA were lower in lean-NAFLD than in overweight-obese NAFLD.

Results of blood examination

The differences in blood examination results among the four groups are shown in Table 3. Both WBC and RBC of NAFLD patients were higher than their controls, and overweight-obese NAFLD had the highest levels among the four groups. This phenomenon was also observed in subtypes of WBC: overweight-obese and lean-NAFLD ranked first and second, respectively in counts of NAUT, LYMPH, MONO and EO. However, the same amount of BASO (P = 0.217) was observed in the four groups. Meanwhile, RBC, HGB and HCT increased with the development of NAFLD.

Association with diseases

Table 4 provides the odd ratios (ORs) from multivariate-adjusted logistic regression analysis for diabetes, hypertension, hyperlipidemia and MetS. We calculated crude ORs and adjusted ORs, which wee adjusted by confounding factors, including age, sex, BMI, smoking, alcohol consumption and physical activity status.

NAFLD significantly added to the risks of almost all chronic diseases: central obesity (OR = 1.97, 95%CI: 1.38-2.80), hyperlipidemia (OR = 1.37, 95%CI: 1.04-1.80), hypertension (OR = 1.37, 95%CI: 1.04-1.80), diabetes (OR = 1.68, 95%CI: 1.08-2.62) and MetS (OR = 2.34, 95%CI: 1.65-3.31). Lean-NAFLD was an independent risk factor for these diseases, except for hyperlipidemia (OR = 1.29, P = 0.30) when NAFLD patients were compared with controls in lean groups. Moreover, NAFLD cases were more likely to have central obesity than lean-controls. Hyperlipidemia, hypertension and diabetes were not more serious in overweight-obese NAFLD patients than overweight-obese controls. NAFLD patients had

Table 2 Clinical biochemical characteristics of non-alcoholic fatty liver disease and controls

	Lean-NAFLD		Overweight-obese-NAFLD		*P* value
	Controls	NAFLD	Controls	NAFLD	
FBG (mmol/L)	5.30 ± 1.36	5.91 ± 1.85[a]	5.52 ± 1.31[b]	5.76 ± 1.42[a]	< 0.01
Fasting insulin (IU/mL)	6.42 ± 3.76	8.27 ± 3.93[a]	7.28 ± 4.33	10.84 ± 5.28[a,c]	< 0.01
HOMA-IR	1.55 ± 0.97	2.16 ± 1.42[a]	1.87 ± 1.35[b]	2.73 ± 1.53[a,c]	< 0.01
TC (mmol/L)	4.86 ± 0.93	5.20 ± 1.01[a]	5.00 ± 0.90[b]	5.10 ± 0.92	< 0.01
TG (mmol/L)	1.18 ± 1.13	1.71 ± 1.23[a]	1.40 ± 1.01[b]	1.83 ± 1.19[a]	< 0.01
HDL-C (mmol/L)	1.58 ± 0.35	1.45 ± 0.30[a]	1.44 ± 0.30[b]	1.35 ± 0.27[a,c]	< 0.01
LDL-C (mmol/L)	2.90 ± 0.79	3.12 ± 0.78[a]	3.09 ± 0.77[b]	3.13 ± 0.78	< 0.01
ALT (U/L)	18.32 ± 13.85	21.61 ± 11.92[a]	21.10 ± 16.13[b]	27.23 ± 18.25[a,c]	< 0.01
AST (U/L)	19.27 ± 6.85	21.07 ± 9.26[a]	20.23 ± 11.10	22.37 ± 9.87[a]	< 0.01
ALP (U/L)	64.34 ± 18.16	71.96 ± 20.49[a]	66.94 ± 21.26	70.00 ± 18.47[a]	< 0.01
GGT (U/L)	26.11 ± 27.99	35.76 ± 34.92[a]	33.42 ± 33.95[b]	47.26 ± 48.44[a,c]	< 0.01
UA (μmol/L)	280.04 ± 82.31	303.30 ± 86.72[a]	307.59 ± 85.54[b]	340.60 ± 92.68[a,c]	< 0.01
BUN (mmol/L)	5.00 ± 1.24	5.15 ± 1.25	5.16 ± 1.19	5.14 ± 1.24	0.15
CRE (μmol/L)	69.26 ± 14.37	70.34 ± 13.84	71.72 ± 14.18[b]	74.53 ± 14.48[c]	< 0.01

[a]$P < 0.05$ *vs* control in same weight group; [b]$P < 0.05$ *vs* Lean-control group; [c]$P < 0.05$ *vs* Lean-NAFLD group. NAFLD: Non-alcoholic fatty liver disease; FBG: Fasting blood glucose; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transferase; UA: Serum uric acid; BUN: Blood urea nitrogen; CRE: Serum creatinine.

Table 3 Hematological comparison between non-alcoholic fatty liver disease and controls among four groups

	Lean-NAFLD		Overweight-obese-NAFLD		*P* value
	Controls	NAFLD	Controls	NAFLD	
WBC (10^9/L)	5.73 ± 1.46	6.40 ± 1.44[a]	6.25 ± 1.58[b]	6.72 ± 1.66[a,c]	< 0.01
NEUT (10^9/L)	3.28 ± 1.12	3.70 ± 1.04[a]	3.62 ± 1.19[b]	3.88 ± 1.25[a,c]	< 0.01
LYMPH (10^9/L)	1.96 ± 0.52	2.14 ± 0.56[a]	2.10 ± 0.57[b]	2.26 ± 0.60[a,c]	< 0.01
MONO (10^9/L)	0.36 ± 0.14	0.39 ± 0.13[a]	0.39 ± 0.16[b]	0.42 ± 0.15[a]	< 0.01
EO (10^9/L)	0.09 ± 0.09	0.13 ± 0.14[a]	0.11 ± 0.10	0.14 ± 0.13[a]	< 0.01
BASO (10^9/L)	0.01 ± 0.04	0.01 ± 0.03	0.01 ± 0.03	0.02 ± 0.03	0.22
RBC (10^{12}/L)	4.50 ± 0.41	4.76 ± 0.42[a]	4.72 ± 0.48[b]	4.96 ± 0.44[a,c]	< 0.01
HGB (g/L)	136.13 ± 15.24	145.87 ± 16.44[a]	143.30 ± 17.05[b]	152.19 ± 14.22[a,c]	< 0.01
HCT (%)	41.36 ± 4.11	44.09 ± 4.41[a]	43.40 ± 4.55[b]	45.68 ± 3.90[a,c]	< 0.01

[a]$P < 0.05$ *vs* control in same weight group; [b]$P < 0.05$ *vs* Lean-control group; [c]$P < 0.05$ *vs* Lean-NAFLD group. NAFLD: Non-alcoholic fatty liver disease; WBC: White blood cell count; NEUT: Neutrophils count; LYMPH: Lymphocytes count; MONO: Monocytes count; EO: Eosinophils count; BASO: Basophils count; RBC: Red blood cell count; HGB: Hemoglobin; HCT: Hematocrit value.

higher risk for MetS (OR = 1.89, 95%CI: 1.29-2.77; OR = 2.17, 95%CI: 1.17-4.05) than overweight-obese and lean-controls.

DISCUSSION

In the current study, we classified the patients with NAFLD as lean- and overweight-obese NAFLD. The prevalence of NAFLD was 18.33% in the lean group and 72.90% in the overweight-obese group. There was a male predominance of NAFLD in lean and overweight-obese individuals (28.48% *vs* 54.48%, and 51.41% *vs* 72.55%, respectively). Compared with controls in the same weight group, both overweight-obese and lean-NAFLD patients had higher levels of BMI, WC, VAI, blood pressure, glucose, dyslipidemia, liver enzymes (ALT, AST, ALP and GGT) and renal function parameters (UA, BUN and GREA). NAFLD was clearly associated with dysfunctional fat and adipose tissue IR, which worsened glucose and fat metabolic status, resulting in pathoglycemia and dyslipidemia[20,21]. NAFLD also added to the risk of chronic kidney disease (CKD)[22] by increasing micro-albuminuria and decreasing glomerular filtration rate[23]. Xu *et al*[24] also found that age, gender, WC, blood pressure, dyslipidemia were significantly associated with NAFLD in a prospective 5-year follow-up of non-obese (BMI < 25 kg/m^2) Chinese subjects. The prevalence of NAFLD almost doubled in the 5-year period. Lean-NAFLD is often asymptomatic and has lower clinical biochemical indicators. A summary of studies on NAFLD stated that the features of lean-NAFLD were different from country to country[25]. Thus, it is difficult to detect and treat in its early stages. It has been proven to be an unrecognized clinicopathological entity and a frequent cause of cryptogenic liver disease[12]. More efforts should be made to halt or reverse the progress of NAFLD in non-obese individuals[14].

The WBC and its subtype cells (NEUT, LYMPH, MONO, EO and BASO) were higher in NAFLD than in controls in both weight groups, and they were even high-

Table 4 Odds ratios of non-alcoholic fatty liver disease compared with controls

	[1]OR (95%CI)	[2]OR (95%CI)
All NAFLD *vs* all controls		
Hyperlipidemia	2.19 (1.81-2.65)	1.37 (1.04-1.80)
Hypertension	2.98 (2.37-3.74)	1.49 (1.10-2.03)
Diabetes	2.97 (2.14-4.11)	1.68 (1.08-2.62)
MS	6.29 (4.78-8.28)	2.34 (1.65-3.31)
Central obesity	6.56 (5.16-8.33)	1.97 (1.38-2.80)
Lean-NAFLD *vs* lean controls		
Hyperlipidemia	1.87 (1.28-2.73)	1.29 (0.80-2.09)
Hypertension	3.26 (2.08-5.10)	1.72 (1.00-2.96)
Diabetes	3.87 (2.11-7.08)	2.47 (1.14-5.35)
MS	5.38 (2.66-10.89)	3.19 (1.38-7.35)
Central obesity	2.37 (1.44-3.90)	2.17 (1.17-4.05)
Overweight-obese NAFLD *vs* overweight-obese controls		
Hyperlipidemia	1.61 (1.22-2.12)	1.36 (0.96-1.91)
Hypertension	1.64 (1.20-2.24)	1.33 (0.92-1.93)
Diabetes	1.87 (1.21-2.91)	1.34 (0.80-2.27)
MS	2.43 (1.75-3.38)	1.89 (1.29-2.77)

[1]OR: Crude OR; [2]OR: Adjusted by age, sex, body mass index, smoking, alcohol consumption and physical activities. NAFLD: Non-alcoholic fatty liver disease; OR: Odds ratio; CI: Confidence interval; MS: Metabolic syndrome.

er in overweight-obese NAFLD than in lean-NAFLD. WBC is a marker of inflammation, and we observed higher levels of WBC and its subtypes in the metabolic disorders[26]. Its count was independently associated with the presence of NAFLD regardless of classical cardiovascular risk factors and other components of metabolic syndrome. Hepatic steatosis is not only a focal fat deposition in the liver, but also a systemic inflammation[27]. RBC, HGB and HCT have a similar rising trend for WBC in NAFLD. These hematological parameters were strongly associated with the prevalence of IR, cerebrovascular damage and metabolic syndrome[28,29]. High HGB, HCT and RBC significantly added to the risk of NAFLD[30]. Serum hemoglobin, which may have a significant predictive value for NAFLD, is an antioxidant, binding to free hemoglobin and inhibiting oxidative injury. Inflammation and oxidative injury both contribute to NAFLD. Thus, NAFLD patients have higher levels of hemoglobin than normal controls[30,31]. The increase of HCT, a decisive factor of blood viscosity, is always followed by a decrease in blood flow rate, leading to an insufficient glucose supply to the muscles, and subsequently IR[32]. Meanwhile, insulin may stimulate erythropoiesis through its growth-promoting effect and increase HCT[33]. Insulin-like growth factor-1 (IGF-1) can stimulate erythropoiesis, and enhance the synthesis of plasma protein in endocrine manner[26]. The above mechanism may be involved in the association between abnormality in blood examination results and NAFLD; however, we could not find any evidence for this phenomenon, and no full-scale blood examination including all parameters has been reported. More research is required to clarify this point.

We also found that NAFLD patients had higher risks for hyperlipidemia, hypertension, diabetes and MetS than the controls. A large number of studies have shown that NAFLD often progresses to dyslipidemia, hypertension, diabetes, cardiovascular disease and CKD, and increases all-cause mortality in many countries[34-39]. Central obesity and IR play important roles in the development of NAFLD[40]. Hepatic enzymes (ALT, AST and GGT) may also contribute to the development of diabetes, and ALT and GGT can predict type 2 diabetes, independent of the degree of adiposity. ALT appears to be positively associated with IR and gluconeogenesis, and reflects inflammation, which impairs insulin signaling in the liver and systemically[41]. ALT is also associated with endothelial dysfunction and predicts coronary heart disease[42]. GGT is an enzyme responsible for extracellular catabolism and may be linked to greater oxidative stress, which is implicated in IR[43]. In summary, NAFLD patients, especially those with elevated liver enzymes, have a higher risk of diabetes and other metabolic disorders.

Lean-NAFLD patients tended to have more visceral adiposity than lean-controls in our study, which can be used to measure the hepatic lipid content. The accumulation of lipid exposes the liver to high concentrations of free fatty acids and triglycerides, resulting in impairment of hepatic metabolic processes. Intrahepatic triglycerides are positively associated with the amount of visceral fat, and a strong negative correlation was observed between triglycerides and systemic insulin sensitivity[44]. Visceral adiposity produces inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF), leading to more serious IR[45]. Liver histology and most cardiometabolic abnormalities can be predicted by the adipose IR index[46].

Gene mutation may also contribute to NAFLD development[47] because of its obvious familial inheritance[48]. A missense mutation in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene (also called adiponutrin), resulted in an approximately 2-fold higher hepatic TG content and an OR of 3.26 for the development of NAFLD. Its relation with hepatic TG content and NAFLD has been identified in many studies[47]. Microbiota perturbation in the gastrointestinal tract may be important in the progression of NAFLD, particularly its role in obesity, insulin resistance and inflammation[49]. In animal models, supplement of lactobacillus fermentum can improve IR, blood lipid metabolism and ameliorate NAFLD[50,51]. Its interaction with he diet is another critical point for this disease[49]. NAFLD is a multifactorial disease, with complex clinical characteristics[31]. More research should be carried out to explore the pathogenesis of NAFLD and associated metabolic disorders.

Chinese people have their own lifestyle and genetic characteristics, which are different from Westerners[52], and are therefore more likely to have lean-NAFLD. Although many studies focused on lean-NAFLD, none of them has demonstrated the harm of NAFLD in lean and overweight-obese individuals. In addition, there is no population-based study in north China. With the increasing prevalence of NAFLD in lean individuals[53], it is essential to distinguish lean from overweight-obese

NAFLD, so that specific treatment can be provided to halt or prevent the development of NAFLD.

In summary, our data provided the estimated prevalence rate of lean-NAFLD in a Chinese population, and its metabolic characteristics compared with overweight-obese patients. The lean-NAFLD group had lower levels of blood glucose, blood pressure, hyperlipidemia, IR, blood cell count and HGB than the overweight-obese NAFLD group. Normal weight individuals are more likely to have diabetes, hypertension and MetS if they have NAFLD. Thus, it is a more dangerous condition than overweight-obese NAFLD.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) has become an important public health issue and obesity is an established risk factor for its development. However, it can also be observed in normal weight individuals, which is called lean-NAFLD. Chinese people have lower body mass index, but a similar prevalence of NAFLD with Western people. We suspected that lean-NAFLD was more serious and has different clinical characteristics in Chinese population.

Research frontiers

Lean-NAFLD has become more and more common, as shown in epidemical studies. It has its own metabolic characteristics and is a major cause of cryptogenic liver disease in normal weight population, when compared with obesity patients. In this study, the authors demonstrated that lean-NAFLD may have different disease status from obese-NAFLD and may be a risk factor for metabolic disorder in lean individuals.

Innovations and breakthroughs

Recent reports have highlighted the importance of obese-NAFLD in the development of metabolic disorders, including diabetes, hypertension and hyperlipidemia. This is the first study to report that NAFLD in lean individuals is a more serious condition than in obese ones, because it adds risk for metabolic diseases. Furthermore, the authors suggested that lean-NAFLD may have different biochemical indexes and blood cell examination results.

Terminology

This study suggests that lean-NAFLD is a dangerous condition, and significantly adds risk for diabetes, hypertension and metabolic syndrome.

Peer review

The authors provided a detailed survey to study the prevalence and disorders of metabolism and blood cell examination in lean-non-alcoholic fatty liver disease (lean-NAFLD). The results of their comparative analysis are clearly presented. It is noticeable that NAFLD subjects with normal weight are more likely to have diabetes, hypertension and metabolic syndrome. Lean-NAFLD has its own metabolic characteristics, such as lower blood glucose, blood pressure, hyperlipidemia, insulin resistance, blood cell count and hemoglobin, which are different from overweight-obese patients. Overall, this paper presents a timely and useful survey of lean-NAFLD features based on the available clinical parameters.

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RETROSPECTIVE COHORT STUDY

Risk factors for early rebleeding and mortality in acute variceal hemorrhage

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Abstract

AIM: To investigate the risk factors for 6-wk rebleeding and mortality in acute variceal hemorrhage (AVH) patients treated by percutaneous transhepatic variceal embolization (PTVE).

METHODS: A retrospective cohort study of AVH patients who had undergone PTVE treatment was conducted between January 2010 and December 2012. Demographic information, medical histories, physical examination findings, and laboratory test results were collected. The PTVE procedure was performed as a rescue therapy for patients who failed endoscopic and pharmacologic treatment. Survival analysis was estimated using the Kaplan-Meier method and compared using the log-rank test. The multivariate analysis was performed using the Cox regression test to identify independent risk factors for rebleeding and mortality.

RESULTS: One hundred and one patients were included; 71 were males and the average age was 51 years. Twenty-one patients rebled within 6 wk. Patients with high-risk stigmata, PTVE with trunk obliteration, and a hepatic vein pressure gradient (HVPG) $\geqslant$ 20 mmHg were at increased risk for rebleeding (OR = 5.279, 95%CI: 2.782-38.454, P = 0.003; OR = 4.309, 95%CI: = 2.144-11.793, P < 0.001; and OR = 1.534, 95%CI: 1.062-2.216, P = 0.022, respectively). Thirteen patients died within 6 wk. A model for end-stage liver disease (MELD) score $\geqslant$ 18 and an HVPG $\geqslant$ 20 mmHg were associated with 6-wk mortality (OR = 2.162, 95%CI: 1.145-4.084, P = 0.017 and OR = 1.423, 95%CI: 1.222-1.657, P < 0.001, respectively).

CONCLUSION: MELD score and HVPG in combination allow for early identification of patients with AVH who are at substantially increased risk of death over the short term.

Key words: Acute variceal hemorrhage; Percutaneous transhepatic variceal embolization; Hepatic vein pressure gradient

Core tip: Acute variceal hemorrhage (AVH) is a medical emergency with a 20% mortality rate at 6 wk. Percutaneous transhepatic variceal embolization (PTVE) is a rescue therapy for endoscopic variceal ligation failure. Here we present a retrospective study to determine the risk factors for 6-wk rebleeding and mortality in AVH patients who have undergone PTVE. Patients with a model for end-stage liver disease score $\geqslant$ 18 and an HVPG $\geqslant$ 20 mmHg are at increased risk of death within 6 wk of an acute variceal bleeding episode. A transjugular intrahepatic portosystemic shunt or liver transplantation should be considered for this high-risk group.

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INTRODUCTION

Gastroesophageal varices are present at diagnosis in nearly one-half of patients with cirrhosis. Variceal hemorrhage carries high rebleeding and mortality rates. In patients with esophageal varices (EV), the combination of endoscopic variceal ligation (EVL) and pharmacologic treatment is recommended as the standard of care for prevention of rebleeding[1]. Mortality with each episode of acute variceal hemorrhage (AVH) has decreased to the current level of approximately 20%[2]. A previous report showed that early rebleeding rate ranges from 30% to 40% within the first 6 wk, and is significantly associated with the risk of death within 6 wk[3]. Thus, research assessing the value of various risk factors in patients with AVH is important in an effort to identify the group of patients at high risk for rebleeding and mortality. A transjugular intrahepatic portosystemic shunt (TIPS) or liver transplantation should be considered for this high-risk group.

Percutaneous transhepatic variceal embolization (PTVE) was introduced in 1974 by Lunderquist *et al*[4] for the management of portal hypertension and EV. PTVE is a safe, easy to perform, and effective treatment for the control of AVH[5]. In the modified PTVE procedure, we used 2-octyl cyanoacrylate as an embolic material to obliterate the EV and peri-esophageal collaterals and feeding vessels[6]. Using multi-detector row computed tomography, a study showed that 2-octyl cyanoacrylate permanently retained in the para- and peri-varices in the vessels without a time-dependent decrease[7]. It is important to continue the obliteration of the feeding vessels and prevent the relapse of esophageal-gastric varices. The modified PTVE technique has been confirmed as an effective and safe method for the management of recurrent gastroesophageal varices and rebleeding compared with endoscopy therapy[8,9]. The combination of PTVE and EVL is more effective than EVL alone in the prevention and treatment of recurrent EV and rebleeding[10]. Thus, PTVE may be used as a rescue therapy for patients who fail endoscopic and pharmacologic treatment.

Some studies have identified the predictors of early rebleeding and mortality after initial EV bleeding in cirrhotic patients with endoscopic treatment[11-16]; however, no study has addressed the association between the predictors of outcome and mortality in cirrhotic patients with EV bleeding treated by PTVE. Studies assessing the value of various risk factors in patients with AVH are important as risk factors may offer a useful means of selection for entry into liver transplantation or they may identify a group of patients with a very high mortality. The early use of TIPS in patients with cirrhosis and at high risk for variceal bleeding was associated with marked and significant reductions in treatment failure and mortality[13].

The aim of this study was to identify the risk factors for rebleeding and mortality in cirrhotic patients admitted to a hospital with a first variceal bleed and treated by PTVE as a rescue therapy after failed endoscopic variceal ligation within 6 wk.

MATERIALS AND METHODS

Patients

Consecutive patients with liver cirrhosis and AVH admitted to our hospital, a tertiary center, were retrospectively analyzed between January 2010 and December 2012. The inclusion criteria were as follows: (1) cirrhosis confirmed by full clinical history, physical examination, laboratory testing, and imaging examinations; (2) patients admitted to hospital within 24 h after the symptom onset, with AVH diagnosed by upper gastrointestinal endoscopy when varices were bleeding actively or showed stigmata of recent bleeding and/or if fresh blood was observed in the stomach and varices were the only potential source of bleeding; and (3) PTVE was performed as a rescue therapy in patients who had uncontrolled severe bleeding or recurrent bleeding from EV during and after band ligation.

The exclusion criteria were as follows: (1) concomitant hepatic cell cancer (HCC) or other cancers; (2) severe hypertension, coronary heart disease, cardiopulmonary insufficiency, or chronic renal insufficiency; (3) previous TIPS placement or endoscopic treatment of varices by sclerotherapy or band ligation; and (4) insufficient data on survival or incomplete medical records.

Therapeutic interventions for variceal bleeding

When admitted to our hospital, all patients were managed by fluid resuscitation and an infusion of vasoactive drugs (octreotide or somatostatin), and the infusion was continued for a total of 3-5 d. Prophylactic oral norfloxacin (400 mg, twice a day) or intravenous ciprofloxacin (1 g, once a day) was administered for 7 d. Sengstaken-Blakemore balloon tamponade was used if necessary. Patients with hemodynamic instability or a significant drop in the hemoglobin level (< 8 g/dL) were given packed red blood cell (PRBC) transfusions to a hemoglobin of 8 g/dL. Upper gastrointestinal endoscopy to identify the source of bleeding was performed on all patients within 24 h of presentation; endoscopic variceal band ligation was performed if the source of gastrointestinal bleeding was believed to be from EV. If band ligation was not feasible because blood obscured the visual field, or if band ligation failed to control bleeding, the PTVE procedure was performed under radiologic guidance, as described previously[6]. The wedged hepatic venous pressure (WHVP) and the free hepatic venous pressure (FHVP) were measured before PTVE, and the hepatic venous pressure gradient (HVPG) was calculated (HVPG = WHVP - FHVP). Propranolol with or without isosorbide mononitrate was used for prevention of recurrent bleeding if patients had no contraindications.

Variable definitions and data collection

Patient outcome was obtained from hospital records and telephone contacts. We included only the first episode for each patient. The primary end point of the study was the first episode of recurrent variceal bleeding 6 wk after the procedure. The secondary end point was death due to primary liver disease in 6 wk. Rebleeding was defined according to the Baveno criteria as recurrence of bleeding evidenced by new melena or hematemesis, requirement for > 2 units of PRBCs in a 24 h time period, and hemodynamic instability[17]. Time zero was defined as the day of the PTVE procedure.

Demographic information, medical history, physical examination with vital signs, documentation of etiology of liver disease and presenting clinical symptoms, ascites, encephalopathy, and Child-Turcotte-Pugh (CTP) classification were collected. The number of blood units transfused within 72 h of admission was recorded. The grade of ascites was based on the definitions of the International Ascites Club[18].

Blood for laboratory testings, including complete blood count, prothrombin time, serum creatinine, total bilirubin, serum albumin, serum sodium, aspartate aminotransferase, and alanine aminotransferase levels was also drawn on the first day of variceal bleeding.

Findings at endoscopy were documented, including sites of varices, stage of EV, presence of active bleeding, and stigmata of high-risk varices. The stage of variceal size was based on the general rules established by the Japanese Research Society for Portal Hypertension[19]. High-risk varices were defined as the presence of an adherent clot or white nipple or red signs on varices (cherry red spot, red wale sign, or hematocystic spots).

The filling range of cyanoacrylate in EVs and the feeding vessels by PTVE was based on the following definitions: (1) complete obliteration, with at least 3 cm of the lower EVs and peri- and para-EVs, as well as the adventitial plexus of the gastric cardia and fundus filled with cyanoacrylate; (2) partial obliteration, with the varices surrounding the gastric cardia, fundus, and the feeding vessels being obliterated with cyanoacrylate, but without reaching the lower EVs; and (3) trunk obliteration, with the main branch of the left gastric vein being filled with cyanoacrylate, but without reaching the varices surrounding the gastric cardia or fundus[7].

MELD scores were calculated according to the following formula: MELD score = 0.957 × ln (creatinine mg/dL) + 0.378 × ln (bilirubin mg/dL) + 1.120 × ln (INR) + 6.43.

Statistical analysis

Continuous data are expressed as mean ± SD, unless specified otherwise. Descriptive statistics (number and percentages) were used to describe discrete data. Survival analysis was estimated using the Kaplan-Meier method and compared using the log-rank test. The multivariate analysis was performed using the Cox regression test to identify independent risk factors for rebleeding and mortality. The Statistical Package for Social Sciences (version 17.0; SPSS, Inc., Chicago, IL, United States) was used, and $P < 0.05$ was regarded as significant.

RESULTS

Between January 2010 and December 2012, 137 cirrhotic patients with AVH underwent PTVE as rescue treatment; 36 patients were excluded from the analysis because of HCC ($n = 5$), technical failures ($n = 4$), previous placement of TIPS or endoscopic treatment ($n = 23$), and incomplete medical records ($n = 4$). Therefore, the number of patients who met the inclusion criteria and were analyzed in the current study was 101. The gastric coronary vein was the main blood vessel for EV in 89 patients. Forty-six patients had varying degrees of contribution from the short gastric and posterior gastric veins. All of the feeding vessels were obliterated with cyanoacrylate. Sengstaken-Blakemore balloon tamponade was used in five patients. Propranolol with or without isosorbide mononitrate was used in 94 patients; the other seven patients did not use propranolol or isosorbide mononitrate because of contraindications (glaucoma, $n = 2$; sinus bradycardia < 50 bpm, $n = 2$; arterial hypotension with systolic pressure < 85 mmHg, $n = 2$; and asthma, $n = 1$). The clinical characteristics of the patients are shown in Table 1.

Risk factors for rebleeding within 6 wk following PTVE treatment

Twenty-one (20.8%) patients rebled within 6 wk of the PTVE procedure. Recurrent bleeding occurred in a range of 3-32 d following PTVE. Among 21 patients with rebleeding, 5 had bleeding from EVL-induced ulcers, 12 from EV, 3 from gastric varices, and 1 from an unknown site. High-risk stigmata of variceal bleeding, PTVE with trunk obliteration, and an HVPG ⩾ 20 mmHg were independent risk factors for rebleeding as revealed by the Kaplan-Meier method. Figure 1 shows survival curves according to independent predictor variables. In multivariable analyses using Cox regression, high-risk stigmata of variceal bleeding, the obliteration range of PTVE, and an HVPG ⩾ 20 mmHg were significantly associated with the risk of rebleeding; high-risk stigmata of variceal bleeding was the variable with the highest odds ratio (OR = 5.279; 95%CI: 2.782-38.454; Table 2).

Risk factors for 6-wk mortality after PTVE

Thirteen (12.9%) patients died within the 6-wk follow-up period. Among these patients, six died of uncontrolled EV bleeding, five of hepatic failure, one of hepatorenal syndrome, and one of hepatic encephalopathy. Cox regression analysis revealed that the MELD score and HVPG were significantly associated with 6-wk mortality after PTVE (Table 3). Figure 2 shows the survival curves according to independent predictor variables. Stratification of patients according to MELD score (MELD ⩾ 18 or MELD < 18) revealed a significant increase in 6-wk mortality after PTVE between patients with MELD scores ⩾ 18 or < 18 ($P = 0.008$; Figure 2A). The HVPG

Table 1 Clinical and biological characteristics of the study population *n* (%)

Characteristic	*n* = 101
Gender (male/female)	71 (70.3%)/30 (29.7%)
Age (yr)	51 ± 12
Etiology of liver disease *n* (%)	
Viral (HBV and/or HCV)	76 (75.2)
Alcohol	19 (18.8)
Others	6 (5.9)
Clinical presentation of bleeding *n* (%)	
Melena	22 (21.8)
Hematemesis	32 (31.7)
Both melena and hematemesis	47 (46.5)
Systolic blood pressure at presentation (mmHg)	114 ± 21
Bleeding source *n* (%)	
Esophageal varices	85 (84.2)
Gastric varices	16 (15.8)
High risk stigmata of variceal bleeding (yes/no)	89 (88.1)/12 (11.9)
Active variceal bleeding at endoscopy (yes/no)	27 (26.7)/74 (73.3)
Hemoglobin (g/dL)	9.9 ± 2.4
White blood cells (10^9/L)	4.78 ± 3.55
Platelets (10^9/L)	111 ± 102
Aspartate aminotransferase level (U/L)	39 ± 15
Alanine aminotransferase level (U/L)	29 ± 15
Serum sodium level (mmol/L)	132 ± 11
Serum creatinine (μmol/L)	57 ± 18
Albumin (g/L)	34 ± 6
Total bilirubin (μmol/L)	24.5 ± 12.3
Prothrombin time (s)	16.7 ± 2.3
Presence of ascites *n* (%)	
0	47 (46.5)
I	31 (30.7)
II	18 (17.8)
III	5 (5.0)
Requiring blood transfusion within 72 h (yes/no)	38 (37.6)/63 (62.4)
Units of PRBCs transfused within 72 h	6 ± 2
HVPG (mmHg)	18 ± 4
Obliteration range of PTVE *n* (%)	
Complete	63 (62.4)
Partial	21 (20.8)
Trunk	17 (16.8)
Child-Turcotte-Pugh classification *n* (%)	
A	15 (14.9)
B	61 (60.4)
C	25 (24.8)
MELD score	15 ± 5

PRBCs: Packed red blood cells; HVPG: Hepatic vein pressure gradient; PTVE: Percutaneous transhepatic variceal embolization.

was also significantly associated with 6-wk mortality after PTVE ($P < 0.001$; Figure 2B). Interestingly, CTP class (A *vs* B/C) was not predictive of mortality.

Adverse effects

Adverse effects were observed in 21 (20.8%) patients following PTVE. Transient upper abdominal pain ($n = 16$), fever ($n = 14$), and bleeding at the liver puncture site ($n = 3$) developed in patients following PTVE. All of the adverse effects were minor and alleviated by pharmacologic therapy.

DISCUSSION

Specific risk factors that predict early rebleeding and mor-

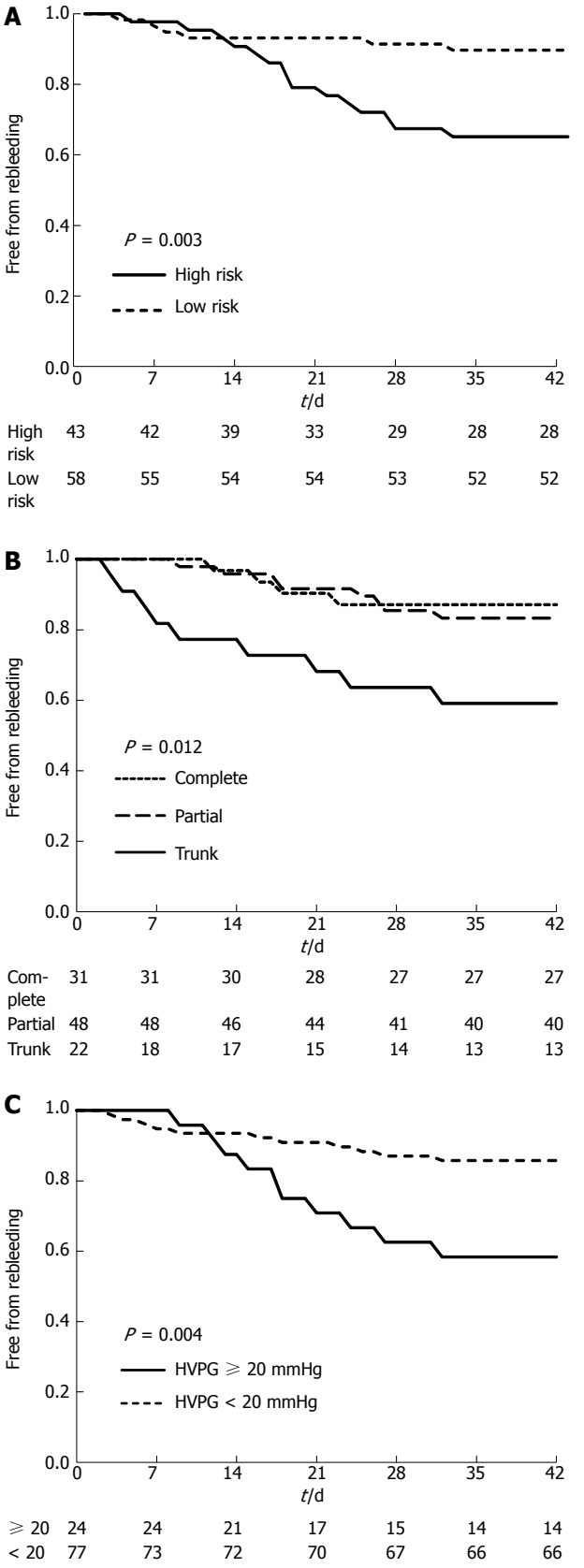

Figure 1 Kaplan-Meier plots showing the cumulative incidence of rebleeding in 6 wk stratified according to (A) risk stigmata of variceal bleeding, (B) obliteration range of percutaneous transhepatic variceal embolization, and (C) hepatic vein pressure gradient. The curves are compared using a log-rank test. PTVE: Percutaneous transhepatic variceal embolization; HVPG: Hepatic vein pressure gradient.

Table 2 Independent risk factors associated with rebleeding as revealed by Cox regression analysis

Variable	B	SE	Wals	*P* value	OR	95%CI	
						Lower	Upper
High risk stigmata of variceal bleeding	4.267	1.435	8.843	0.003	5.279	2.782	38.454
Obliteration range of PTVE	2.068	0.540	14.663	0.000	4.309	2.144	11.793
HVPG	0.428	0.188	5.209	0.022	1.534	1.062	2.216

PTVE: Percutaneous transhepatic variceal embolization; HVPG: Hepatic vein pressure gradient.

Table 3 Independent prognostic factors associated with mortality as revealed by Cox regression analysis

Variable	B	SE	Wals	*P* value	OR	95%CI	
						Lower	Upper
MELD	0.771	0.324	5.652	0.017	2.162	1.145	4.084
HVPG	0.353	0.078	20.691	0.000	1.423	1.222	1.657

MELD: Model for end-stage liver disease; HVPG: Hepatic vein pressure gradient.

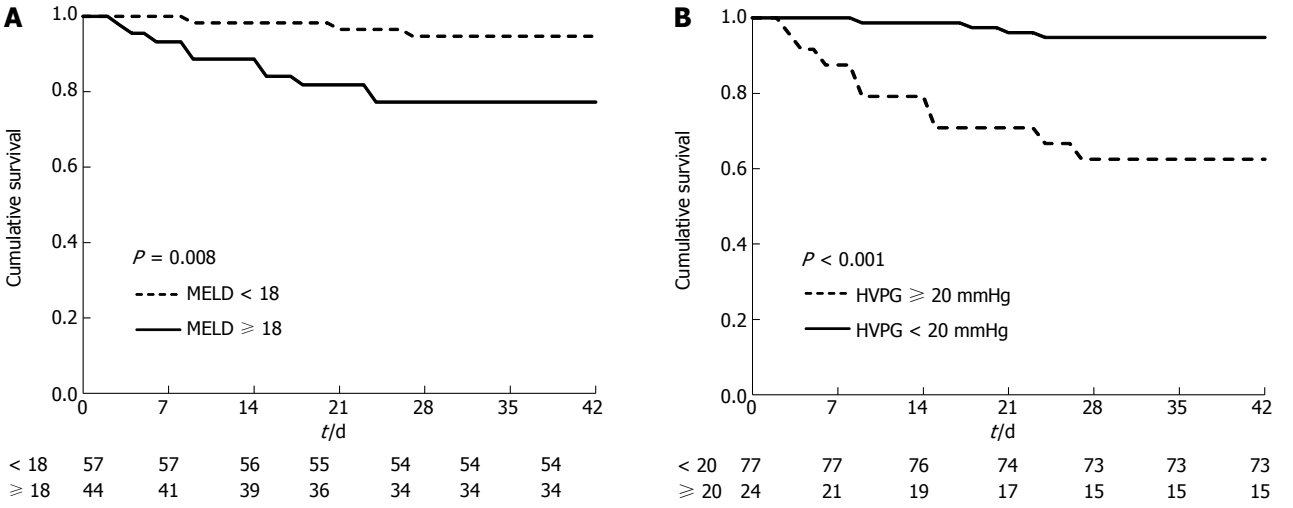

Figure 2 Kaplan-Meier plots showing the cumulative incidence of death in 6 wk stratified according to model for end-stage liver disease (A) and hepatic vein pressure gradient (B). The curves are compared using a log-rank test. MELD: Model for end-stage liver disease; HVPG: Hepatic vein pressure gradient.

tality after variceal bleeding with PTVE treatment have not been studied. In the present study, we found that high-risk stigmata of variceal bleeding, the obliteration range of PTVE, and an HVPG ⩾ 20 mmHg are significantly predictive of 6-wk rebleeding in patients with cirrhosis who are hospitalized with an acute variceal bleed and treated by PTVE as rescue treatment. By complete and permanent obliteration of the lower EVs, peri- and para-EVs, and the adventitial plexus of the gastric cardia and fundus, complete obliteration of PTVE can reduce the risk of variceal recurrence, and prevent bleeding from EV, while varices tend to reoccur over time following partial and incomplete trunk obliteration of PTVE.

Both HVPG measurement and endoscopic features as prognostic indicators of early rebleeding in patients with EVL treatment have been reported in some studies. A retrospective study conducted by Lee *et al*[20] showed that early recurrent hemorrhage in cirrhotic patients is significantly associated with more EV ligations due to the extensive surface area of the mucosal injury and post-banding ulcers. Xu *et al*[21] showed that the severity of varices is one of the main factors affecting early rebleeding after EVL.

The rate of mortality within 6 wk after cessation of initial EV bleeding in our study was 12.9%, which was similar with the lower rates reported in previous studies (range, 8%-46%)[22-25]. Our study also demonstrated that MELD and HVPG are related to early mortality for EV patients following PTVE.

Several studies have investigated the accuracy of the MELD score in predicting mortality after AVH. In a retrospective study of 172 cirrhotic patients admitted for esophageal variceal hemorrhage, Amitrano *et al*[26] showed that patients with an MELD score > 15 had significantly higher mortality at 6 wk than patients with an MELD score ⩽ 15. By regression analysis of 256 patients with AVH in a randomized, prospective trial, Bambha *et al*[27] demonstrated that patients with a MELD score ⩾ 18, those transfused with ⩾ 4 units of packed erythrocytes

within the first 24 h or those being actively bleeding at the time of endoscopy had increased mortality within 6 wk. Based on a study by Suk *et al*[28], the efficacy of HVPG and MELD is excellent for predicting the survival of patients with decompensated liver cirrhosis. Ripoll *et al*[29] showed that MELD was the only predictor of death in decompensated patients based on multivariate analysis. Reverter *et al*[11] developed an MELD-based model that accurately predicts mortality among patients with AVH; MELD values ≥ 19 predicted ≥ 20% mortality, whereas MELD scores < 11 predicted < 5% mortality. MELD also was a significant predictor of mortality for patients with variceal bleeding admitted to intensive care units[30]. Wang *et al*[31] reported that HVPG measurement may help identify a subset of patients with low MELD scores who have a higher mortality. Our study showed that MELD score and HVPG measurement can be used as stratified factors to discern high-risk patients and make a decision to proceed to TIPS or liver transplantation earlier.

Additionally, we found that the etiology of liver disease (alcohol *vs* viral *vs* others), active bleeding at the index endoscopy, volume of blood transfusion in 72 h, and CTP class were not correlated with the risk of rebleeding and mortality based on univariate analyses. These results are somewhat discordant with several other studies focusing on the prognosis of variceal bleeding. In unselected cirrhotic patients, Amitrano *et al*[32] concluded that CTP class C was an independent predictor of 5-d failure; mortality was mainly related to the severity of liver failure. Bambha *et al*[27] demonstrated that patients who received ≥ 4 units of packed erythrocytes within the first 24 h or were actively bleeding at the time of endoscopy had an increased mortality rate within 6 wk.

Differences in patient sampling (percentage of alcoholics and percentage of CTP class C cirrhotic patients), variables recorded, techniques of endoscopic intervention, or dissimilar study end points could explain the discrepancies. In the current study, the source of bleeding (esophagus or stomach) and the activity of bleeding (active or recent) were defined according to the Baveno V consensus[17]. EVL was defined as a primary therapy; patients underwent endoscopy therapy as soon as they were hemodynamically stable and PTVE was a rescue therapy when EVL failed. Furthermore, we only analyzed the first bleeding episode. It is very important to distinguish the first from the subsequent bleeding episodes because associated mortality is different and this may lead to biases in studies when pooling the two types of episodes[33].

Interestingly, we found that the risk factors for rebleeding and mortality were different. Our study therefore suggests that although rebleeding is the major cause of cirrhosis-associated deaths, death is influenced not only by the severity of the bleeding episode itself, but also by the severity of the underlying liver disease and concomitant diseases.

There were two limitations in the current study. First, PTVE is not a standard treatment for AVH. EVL is the recommended form of endoscopic therapy for AVH; rebleeding after EVL may be managed by a second attempt at endoscopic therapy[1]. If rebleeding is severe, PTFE-covered TIPS is likely the best option[34]; however, TIPS is a complex procedure, and in some patients, such as those with variant anatomy, portal vein thrombosis, hepatic vein thrombosis, or pre-existing TIPS, TIPS creation may be extremely difficult[35]. Contraindications and complications of TIPS also restrict its use in cirrhosis patients[36]. Modified PTVE with 2-octyl cyanoacrylate has been confirmed as an effective and safe method for preventing rebleeding of EV and gastric varices[9,10,37]. Variceal embolotherapy during TIPS procedures is a rational approach to reducing recurrent bleeding rates after TIPS placement. According to a recent study, the TIPS plus embolization regimen may reduce the risk of recurrent variceal bleeding during the first 6 months after the TIPS procedure by preventing shunt dysfunction, which may improve liver function and quality of life[38], Therefore, we used PTVE as rescue therapy for EVL failure, and the current study demonstrated the risk factors for rebleeding and mortality after AVH with PTVE treatment.

Second, an absence of standardization at the time of entry also affected the study. Difference in the time of entry could lead to different results. As Burroughs *et al*[39] reported, the starting point for analysis following variceal hemorrhage is an important confounding variable when calculating survival and rebleeding. Changing the starting point for analysis after variceal hemorrhage leads to completely different conclusions. Usually, the entry time is the day patients are admitted to the hospital in studies involving risk factors related to rebleeding and mortality in AVH or EVL-treated patients. However, in the current study, our aim was to determine the risk factors related to rebleeding and mortality after PTVE treatment, so we chose the day of the PTVE procedure as time zero, which avoided rebleeding and mortality before PTVE.

In conclusion, the current study demonstrated that stigmata of variceal bleeding, the obliteration range of PTVE, and an HVPG ≥ 20 mmHg are significant and strong predictors of short-term rebleeding 6 wk after AVH treated by PTVE. We also demonstrated that patients with an MELD score ≥ 18 and an HVPG ≥ 20 mmHg are at increased risk of death within 6 wk of an acute variceal bleeding episode. Together these factors allow for early identification of patients with AVH who are at substantially increased risk of death over the short term. Such patients would also probably benefit from early TIPS or liver transplantation. Furthermore, the PTVE procedure combined with TIPS may improve survival in AVH patients and is worthy of further study.

COMMENTS

Background

Acute variceal hemorrhage (AVH) is a medical emergency with a 20% mortality rate at 6 wk. Recurrent variceal bleeding is very frequent and risk factors for early rebleeding and mortality in AVH patients are ill-defined.

Research frontiers

Research assessing the value of various risk factors for AVH patients is impor-

tant in an effort to identify the group of patients at high risk for rebleeding and mortality. Percutaneous transhepatic variceal embolization (PTVE) is a rescue therapy for endoscopic variceal ligation failure. Specific risk factors that predict early rebleeding and mortality after variceal bleeding with PTVE treatment have not been studied.

Innovations and breakthroughs

In previous studies involving risk factors for early rebleeding in patients who had undergone EVL treatment, it was reported that severity of varices, MELD score, transfusion, and Child class were related to AVH rebleeding and mortality. However, specific risk factors that predict early rebleeding and mortality after variceal bleeding with PTVE treatment have not been studied. In the present retrospective cohort study, we found that high-risk stigmata, PTVE with trunk obliteration, and an HVPG $\geqslant$ 20 mmHg are predictors of variceal rebleeding within 6 wk, and patients with an MELD score $\geqslant$ 18 and an HVPG $\geqslant$ 20 mmHg are at increased risk of death within 6 wk. In combination, these factors allow for early identification of patients with AVH who are at substantially increased risk of rebleeding or death over the short term. Such patients would probably benefit from early TIPS or liver transplantation.

Applications

The current study results suggest that patients with an MELD score $\geqslant$ 18 and an HVPG $\geqslant$ 20 mmHg are at increased risk of death within 6 wk after an acute variceal bleeding episode. Research assessing the value of various risk factors in patients with AVH is important in an effort to identify the group of patients at high risk for rebleeding and mortality. A transjugular intrahepatic portosystemic shunt (TIPS) or liver transplantation should be considered for this high-risk group.

Terminology

Percutaneous transhepatic variceal embolization (PTVE) is the earliest intervention performed for the treatment of intractable variceal bleeding. During PTVE, the portal vein is catheterized by a percutaneous transhepatic approach and the gastric vein feeding the varix is embolized with ethanol, steel coils, or cyanoacrylate glue. The hepatic venous pressure gradient (HVPG) is currently the most commonly used parameter for portal pressure measurement, *i.e.*, the difference between the wedged and free hepatic venous pressures. HVPG represents the gradient between pressures in the portal vein and the intra-abdominal portion of the inferior vena cava.

Peer review

This is an interesting study debating a well-chosen topic. The study adequately addresses several points of an on-going debate regarding AVH, rebleeding risk, and treatment.

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RETROSPECTIVE STUDY

Clinicopathological features of small nonfunctioning pancreatic neuroendocrine tumors

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Abstract

AIM: To present our experiences in studying the clinicopathological features of small nonfunctioning pancreatic neuroendocrine tumors (NF-pNETs).

METHODS: The subjects included 9 patients with NF-pNETs who underwent pancreatectomy between April 1996 and September 2012. The surgical procedure, histopathological findings, and prognosis were assessed.

RESULTS: All tumors were incidentally detected by computed tomography. The median diameter was 10 mm (5-32 mm). One patient was diagnosed with von Hippel-Lindau disease, and the others were sporadic cases. For the histopathological findings, 7 patients were G1; 1 patient was G2; and 1 patient, whose tumor was 22 mm, had neuroendocrine carcinoma (NEC). One patient who had a tumor that was 32 mm had direct invasion to a regional lymph node and 1 patient with NEC, had regional lymph node metastases. Six of the 7 patients with sporadic NF-pNETs, excluding the patient with NEC, had tumors that were smaller than 10 mm. Tumors smaller than 10 mm showed no malignancy and lacked lymph node metastasis.

CONCLUSION: Sporadic NF-pNETs smaller than 10 mm tend to have less malignant potential. These findings suggest that lymphadenectomy may be omitted for small NF-pNETs after further investigation.

Key words: Pancreatic neuroendocrine tumor; Pancreatic neuroendocrine carcinoma; Nonfunctioning; Lymphadenectomy; Treatment

Core tip: We present our experience in studying the clinicopathological features of small nonfunctioning pancreatic neuroendocrine tumors (NE-pNETs). In the present study, six of the 7 patients with sporadic NF-pNETs, excluding the patient with NEC, had small tumors that were less than 10 mm. These small tumors showed no sign of malignancy or lymph node metastasis. Additionally, these cases did not have recurrence, including lymph node and distant metastasis, for more than 10 years after surgery. These findings suggest that small NF-pNETs tend to have less malignant potential and no lymph nodes metastasis. Lymphadenectomy may be omitted in the future for small NF-pNETs after further investigation.

Furukori M, Imai K, Karasaki H, Watanabe K, Oikawa K, Miyokawa N, Taniguchi M, Furukawa H. Clinicopathological

features of small nonfunctioning pancreatic neuroendocrine tumors. *World J Gastroenterol* 2014; 20(47): 17949-17954 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17949.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17949

INTRODUCTION

Pancreatic neuroendocrine tumors (pNETs) are relatively rare, accounting for 1%-2% of all pancreatic neoplasms[1]. Although pNETs progress slowly and have better a prognosis than pancreatic cancer, pNETs have malignant potential, including features of local invasion, lymph node metastasis, and distant metastasis. The appropriate diagnosis and treatment of pNETs are crucial. These tumors are classified into functioning pNETs (F-pNETs), which present with specific symptoms due to excess hormones, and nonfunctioning pNETs (NF-pNETs), which do not present with these symptoms. Because NF-pNETs do not present with specific symptoms, they are often detected as large tumors in the advanced stage, with distant metastasis or invasion to adjacent organs. However, improvements in diagnostic imaging over the last few decades have led to the incidental detection of small NF-pNETs *via* diagnostic imaging for the work-up of other conditions. The incidence of malignancy reportedly increases with larger NF-pNETs[2,3]. However, even small NF-pNETs have malignant potential and may spread to lymph nodes or metastasize to distant sites. Therefore, once NF-pNETs are diagnosed, all cases are considered for surgical resection[4]. The significance of lymph node metastasis in the NF-pNETs has been reported[5-9]; the prognosis is poor with a 5-year survival of 49.4%, even after resection, in cases with lymph node metastasis[7]. Therefore, lymphadenectomy, in addition to tumor resection, is recommended when the tumor is malignant or when lymph node metastasis is suspected. However, there are no standard criteria for lymphadenectomy when small, asymptomatic, and incidentally detected NF-pNETs are identified. The inclusion of lymphadenectomy during surgery for NF-pNETs remains controversial.

In the present study, we report 9 cases of NE-pNETs treated at our hospital over the last 16 years.

MATERIALS AND METHODS

Between 1996 and 2012, 26 patients with pNETs underwent pancreatectomy at Asahikawa Medical University Hospital, of whom 9 patients were diagnosed with NF-pNETs and were further investigated. The diagnosis of pNET was established by histopathological examination and immunohistochemical staining of surgical specimens with chromogranin A, synaptophysin, and neuron-specific enolase stain. Tumors were classified as nonfunctioning regardless of the plasma hormone levels or immune activity of the tissue if the patient lacked the clinical symptoms that are typically caused by excess hormones. The patients' medical records were retrospectively reviewed. All patients were pathologically classified according to the criteria established by the WHO 2010 classification of endocrine tumors[4]. An immunohistochemical staining assay for Ki67 was performed for all patients. The Ki67 proliferative index is expressed as a percentage based on the count of Ki67-positive cells in a set of 2000 tumor cells in areas with the highest immunostaining, which was evaluated with the MIBI antibody, and the cases were classified into the following 3 categories: G1 (mitoses/10 HPFs < 2 and/or Ki67 index < 3), G2 ($2 \leqslant$ mitoses/10 HPFs $\leqslant 20$ and/or $3 \leqslant$ Ki67 index $\leqslant 20$), and neuroendocrine carcinoma (NEC) (mitoses/10 HPFs > 20 and/or Ki67 index > 20). The tumor size was defined by the largest diameter of the tumor. A TNM stage group was assigned to each case based on the European Neuroendocrine Tumor Society (ENETS) staging classification for pNETs[10]. The postoperative follow-up included clinical examination, the blood neuron specific γ-enolase (NSE) level, and contrast-enhanced computed tomography (CT) scanning. CT scans were performed every 6 to 12 mo in the first year, then annually thereafter.

RESULTS

In this study, the tumors identified as NF-pNETs accounted for 2.8% of all pancreatic neoplasms (9/220) and for 35% of pNETs (9/26). Table 1 summarizes the clinical features, surgical procedure, histopathological findings, prognosis, WHO classification, and ENETS TNM classification of the 9 patients diagnosed with NF-pNETs. These patients included 3 men and 6 women with a mean age of 67 years (range, 47-75 years) at the time of surgery. One patient with von Hippel-Lindau disease had previously undergone enucleation of the pNETs; the others were sporadic cases. All patients with NF-pNETs were asymptomatic, and none had evidence of distant metastasis. In all cases, the pancreatic tumors were incidentally detected by radiological investigation during evaluations for unrelated conditions. None of the patients had a preoperatively elevated blood level of NSE. Three patients underwent endoscopic ultrasonography-fine needle aspiration (EUS-FNA) and were preoperatively diagnosed with pNETs (No. 2, 6, and 8). All patients underwent surgical resection of the pancreas: 3 patients underwent distal pancreatectomy (DP), 2 patients underwent pylorus-preserving pancreatoduodenectomy (PPPD), 2 patients underwent subtotal stomach-preserving pancreatoduodenectomy (SSPPD), and 2 patients underwent partial resection of the pancreas. R0 resection was performed in all patients, except in 1 patient who underwent partial resection with positive surgical margins (No. 5). Regional lymphadenectomy was performed in 5 of the 9 patients (No. 2, 3, 6, 7, and 8). The median tumor diameter was 10 mm (range, 5-32 mm). All patients, except for the patient with von Hippel-Lindau disease (4 tumors), had a single tumor. Six patients had tumors located in the head

Table 1 Clinical and pathological status of 9 patients with nonfunctioning pancreatic neuroendcrine tumors

No	Age (yr)	Sex	Size (mm)	Location	Number of tumor	EUS-FNA	Preoperative diagnosis	Surgical procedure	Lymphadenectomy	Metastases		Motoses	Ki67/ MiB-1 (%)	WHO classification 2010	TNM classification (ENET)	Prognosis (mo)
										Lymph node	Distant					
1	58	F	32	Ph	1	No	Pancreatic tumor	DP	No	Direct Invasion	No	0	0.2	NET G1	T2N1M0	59 alive
2	73	M	22	Ph	1	No	NET	PPPD	Regional	No	No	1	5.8	NET G2	T2NOMO	39 alive
3	67	F	22	Pb	1	Done	NET G1	DP	Regional	Positive	No	20	20	NEC	T2N1M0	14 alive
4	74	F	10	Pb	1	No	Islet cell tumor	DP	No	No	No	0	1.6	NET G1	T1N0M0	196 alive
5	61	M	10	Pb	1	No	Islet cell tumor	Partial resection	No	No	No	0	0.1	NET G1	T1N0M0	135 alive
6	51	F	9	Ph	1	Done	NET G1	PPPD	Regional	No	No	0	1	NET G1	T1N0M0	64 alive
7	47	F	6 2.1 1.2 1.2	Ph	4	No	NET	SSPPD	Regional	No	No	0	0.9	NET G1	T1N0M0 Stage I	22 alive
8	75	M	6	Ph	1	Done	NET G1	SSPPD	Regional	No	No	0	< 1	NET G1	T1N0M0	20 alive
9	56	F	5	Ph	1	No	Cartinoid	Partial resection	No	No	No	0	0.4	NET G1	T1N0M0	34 alive

EUS-FNA: Endoscopic ultrasonography-fine needle aspiration; Ph: Head of pancreas; Pb: Body of pancreas; DP: Distal pancreatectomy; PPPD: Pylorus-preserving pancreatoduodenectomy; SSPPD: Subtotal stomach preserving pancreatoduodenectomy.

of the pancreas, while 3 patients had tumors located in the body of the pancreas. Seven patients were classified as G1, and 1 patient with a tumor that was 22 mm in diameter was classified as G2. Although 1 patient, with a tumor that was 22 mm in diameter, was diagnosed as G1 by preoperative EUS-FNA, the final diagnosis was neuroendocrine carcinoma (NEC). None of the patients, except two cases, had no lymph nodes metastasis; one with lymph node metastasis had a tumor that was 32 mm in diameter with direct invasion to the regional lymph nodes, and the other had NEC with regional lymph nodes metastasis. Six of the 7 patients with sporadic NF-pNETs had small tumors that were less than 10 mm in size; one patient with NEC had a larger tumor. Tumors that were less than 10 mm in size showed no malignancy, were well differentiated, and lacked lymph node metastasis. Six patients were classified as Stage Ⅰ, 1 patient was classified as Stage Ⅱa, and 2 patients were classified as Stage Ⅲb. With respect to the postoperative complications, three patients had a pancreatic fistula, one patient was classified as Grade B (No. 3), and 2 patients were classified as Grade A (No. 1 and 2) according to the ISGPS criteria. None of the patients in this study had exocrine or endocrine insufficiency. The mean follow-up period was 63 mo (range, 14-196 mo). All of the patients are currently alive without disease recurrence according to radiological imaging.

DISCUSSION

In the present study, we examined the NF-pNETs in 9 patients who underwent pancreatectomy at our institution over the last 16 years. For all of the patients, the tumors were incidentally detected by diagnostic imaging during a work-up for other conditions. Most tumors were small, with a diameter of 5-32 mm (median: 10 mm), and none of the tumors showed evidence of distant metastasis. While the larger tumors tended to be associated with direct invasion of the lymph nodes and lymph node metastases, a high Ki-67 index, and an advanced TNM stage, tumors that were smaller than 10 mm in diameter lacked malignancy and lymph node metastasis.

NF-pNETs are relatively rare, and only 9 patients presented with NF-pNETs at our institution over the last 16 years. In Western nations, pNETs occur at an incidence of 1 per 100000 individuals and represent 1%-2% of all pancreatic neoplasms[1]. Over the last few years, however, this incidence has increased[11,12]. An epidemiological study by NETWork Japan in 2005 estimated that the incidence of pNETs per 100000 individuals is 2.23 patients in Japan. Compared with Western nations, Japan has a 2- to 3-fold higher incidence of pNETs[3]. In total, 30%-50% of all pNETs are nonfunctioning[3,13]; however, because NF-pNETs do not present with characteristic clinical symptoms due

to excess hormones, they often go unnoticed until they are in the advanced stages. Previously, NF-pNETs were often detected as larger tumors that were accompanied by nonspecific pressure symptoms, such as abdominal pain or discomfort; abdominal distension; or a palpable mass in advanced stages with distant metastasis or local invasion. The number of NF-pNETs that have been incidentally detected has increased due to the advances in diagnostic imaging over the last few decades. Compared with other pancreatic tumors, pNETs progress slowly and are associated with a better prognosis. However, they have malignant potential, including local invasion, lymph node metastasis, or distant metastasis. More than half of NF-pNETs are malignant[3,13]. Therefore, most recommendations favor surgical resection for all patients, even for small NF-pNETs[4].

Numerous retrospective studies have previously examined the poor prognosis for NF-pNETs[6,7,14-21]. According to these studies, the predictors of the prognosis for NF-pNETs include the presence of liver metastases and incomplete resection of the tumor.

Several studies have indicated that lymph node metastasis is a poor prognostic factor[5-9]. In addition, Boninsegna *et al*[8] reported that lymph node metastasis is a prognostic factor for the recurrence of malignant pNETs after curative surgery. If malignancy of the tumor or lymph node metastasis is suspected, pancreatic resection with the addition of lymphadenectomy is recommended. It is often difficult to judge preoperatively whether a tumor is benign or malignant, except in patients with distant metastases or local invasion.

The tumor size appears to correlate with the malignant potential of NF-pNETs. Bettini *et al*[2] reported that the chance of malignancy significantly increases when the size of NF-pNETs exceeds 20 mm. A Japanese epidemiological study also found a significant correlation between NF-pNETs that exceed 20 mm in diameter and the presence of distant metastases[3]. Pancreatic resection and prophylactic regional lymphadenectomy are recommended for treating possible malignancy when the tumors exceed 20 mm in diameter[4]. However, several studies have failed to identify a correlation between the tumor size and prognosis[5,13,22,23], and other studies have demonstrated that even tumors smaller than 10 mm can be malignant[24,25]. Therefore, surgical resection is recommended even in small tumors.

Currently, the association between the tumor size and the incidence of lymph node metastasis is controversial. Hashim *et al*[9] reported that there is an increased probability of nodal metastasis when the tumor size is larger than 15 mm. Tsutsumi *et al*[26] reported an increased prevalence of lymph node metastasis in patients with gastrinomas and non-gastrinoma who have tumor sizes of 15 mm or larger. In contrast, Parekh *et al*[27] reported that the tumor size is not associated with lymph node metastasis. A number of studies have reported that the incidences of lymph node metastases for patients with NF-pNETs smaller than 20 mm and 15 mm are 14.4% and 8%, respectively[2,9,26-29]. Over the last few decades, the number of NF-pNETs that are incidentally detected with diagnostic imaging has increased, and compared with symptomatic NF-pNETs, tumors that are incidentally detected have a good prognosis and low risk of malignancy[2,21].

In the present study, one of the 9 patients was diagnosed with von Hippel-Lindau disease, and this patient should be considered separately because the biological properties of sporadic pNETs and hereditary pNETs, such as MEN-1 and von Hippel-Lindau disease, are different with respect to the incidence, number of tumors, and prognosis. One of the 8 patients with sporadic NF-pNETs had NEC with a tumor size of 22 mm. Except for the case with NEC, the direct invasion and metastasis to the lymph nodes was only observed in a relatively large tumor with a diameter size of 32 mm. Tumors smaller than 10 mm in diameter showed no signs of malignancy, were well differentiated, and lacked lymph node metastasis. Additionally, none of the cases had recurrence, including in the lymph nodes or direct metastasis, for more than 10 years after surgery. Lymphadenectomy may be omitted in the future after further investigation of a large number of small NF-pNETs. However, Hashim *et al*[9] reported that even tumors smaller than 10 mm metastasize at a rate of 12%. Additionally, lymphadenectomy is often omitted for small pNETs that are larger than 10 mm in size; the possibility of lymph node metastasis may be underestimated in those cases. Omission of lymphadenectomy needs to be carefully considered with further study. Even when lymphadenectomy is omitted, long-term follow-up is essential because there is a risk of late recurrence. If malignancy is confirmed postoperatively, oncologically appropriate lymphadenectomy must be considered based on the factors that determine the malignant potential, such as the Ki67 index, tumor differentiation status, surgical margin, and vascular invasion such as lymphoductal, neural, and venous[19,20].

In the present study, CgA, PP, and other hormones were not measured; it is important to measure these hormones to identify recurrences during follow-up.

The present study is limited by its small sample size, single institution bias, and retrospective nature. In the future, a larger number of patients at multiple centers should be studied.

In summary, we found that small NF-pNETs tend to have less malignant potential. In the present study, six of 7 cases of sporadic NF-pNETs, except for a case with NEC, were small tumors (smaller than 10 mm diameter). These small tumors showed no evidence of malignancy, were well differentiated, and lacked lymph node metastasis. This finding indicates that lymphadenectomy may be omitted in the future for small NF-pNETs, particularly for those tumors that are incidentally detected after further investigation. When lymphadenectomy is omitted, long-term follow-up is essential, and additional resection should be considered if malignancy is confirmed postoperatively. The tumor size can easily be measured pre-

operatively, and further study is expected to find other factors for predicting the malignant potential of small NF-pNETs.

COMMENTS

Background

Even small NF-Pancreatic neuroendocrine tumors (pNETs) have malignant potential and may spread to lymph nodes or metastasize to distant sites. Therefore, oncologic resection with regional lymphadenectomy is currently recommended. Increasingly smaller NF-pNETs are being identified with improved and more frequent radiological imaging. However, because the clinicopathological features of extremely small NF-pNETs are not yet known, there are no standard criteria for performing a lymphadenectomy when small, asymptomatic NF-pNETs are identified.

Research frontiers

NF-pNETs have malignant potential and may spread to lymph nodes or metastasize to distant sites. However, the clinicopathological features of extremely small NF-pNETs are not yet known. In this study, the authors present their experience with the clinicopathological features of small NF-pNETs (diameters less than 10 mm).

Innovation and breakthroughs

Small NF-pNETs are being identified with improved and more frequent radiological imaging. However, few studies have examined small NF-pNETs with diameters less than 10 mm. In this study, tumors with diameters less than 10 mm showed no evidence of malignancy, were well differentiated, and lacked lymph node metastasis. Additionally, there were no recurrences after the operations, including in the lymph nodes or direct metastasis, for more than 10 years after surgery.

Applications

A previous study reported that the incidence of lymph metastasis is higher for larger tumors. Our findings indicate that lymphadenectomy of small NF-pNETs may be omitted in the future after further investigation of a large number of patients with small NF-pNETs.

Terminology

pNETs are relatively rare disease and progress slowly and are associated with a better prognosis. However, they have malignant potential, including local invasion, lymph node metastasis, or distant metastasis. pNETs are classified into functioning pNETs, which present with specific symptoms due to excess hormones, and nonfunctioning pNETs (NF-pNETs), which do not present with these symptoms.

Peer review

The present manuscript by Furukori *et al* focuses on the need of lymphadenectomy in NF-pNETs < 10 mm and suggests that in these tumors the lymphadenectomy can be omitted. The concept is very challenging.

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RETROSPECTIVE STUDY

Interventional digital subtraction angiography for small bowel gastrointestinal stromal tumors with bleeding

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Abstract

AIM: To retrospectively evaluate the diagnostic efficacy of interventional digital subtraction angiography (DSA) for bleeding small bowel gastrointestinal stromal tumors (GISTs).

METHODS: Between January 2006 and December 2013, small bowel tumors in 25 consecutive patients undergoing emergency interventional DSA were histopathologically confirmed as GIST after surgical resection. The medical records of these patients and the effects of interventional DSA and the presentation and management of the condition were retrospectively reviewed.

RESULTS: Of the 25 patients with an age range from 34- to 70-year-old (mean: 54 ± 12 years), 8 were male and 17 were female. Obscure gastrointestinal bleeding, including tarry or bloody stool and intermittent melena, was observed in all cases, and one case also involved hematemesis. Nineteen patients required acute blood transfusion. There were a total of 28 small bowel tumors detected by DSA. Among these, 20 were located in the jejunum and 8 were located in the ileum. The DSA characteristics of the GISTs included a hypervascular mass of well-defined, homogeneous enhancement and early developed draining veins. One case involved a complication of intussusception of the small intestine that was discovered during surgery. No pseudoaneurysms, arteriovenous malformations or fistulae, or arterial rupture were observed. The completely excised size was approximately 1.20 to 5.50 cm (mean: 3.05 ± 1.25 cm) in maximum diameter based on measurements after the resection. There were ulcerations (n = 8), erosions (n = 10), hyperemia and edema (n = 10) on the intra-luminal side of the tumors. Eight tumors in patients with a large amount of blood loss were treated with transcatheter arterial embolization with gelfoam particles during interventional DSA.

CONCLUSION: Emergency interventional DSA is a useful imaging option for locating and diagnosing small bowel GISTs in patients with bleeding, and is an effective treatment modality.

Key words: Gastrointestinal stromal tumors; Small bowel; Digital subtraction angiography; Interventional; Embolization; Bleeding

Core tip: This study sample is the largest of its kind, and this article includes a detailed report on the patients with small bowel gastrointestinal stromal tumors (GISTs) based on digital subtraction angiography (DSA) visualization. The data indicate that an exact location can be determined and a diagnosis of the small bowel GISTs can be made even in patients undergoing emergency interventional DSA for obscure gastrointestinal bleeding. For cases of severe anemia, these patients

can also tolerate interventional DSA and management. Therefore, interventional DSA is an alternative effective imaging modality for locating and diagnosing small bowel GISTs, even in patients with bleeding.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal (GI) tract. They occur most commonly in the stomach, small intestine, large intestine and esophagus[1-3]. Although small bowel GISTs account for only 20% to 45% of GISTs, in recent years they have reportedly become more common and display more aggressive biobehaviors. Moreover, the recurrence rate of small bowel GISTs is higher than that of other tumors in the stomach or other sites[4-6]. Common symptoms of small bowel GISTs include abdominal pain, abdominal mass, GI bleeding and intestinal obstruction, but these cases are often nonspecific or asymptomatic, especially in cases involving smaller tumors[1-4,6]. The lack of specific clinical manifestations and the location of the tumors make the diagnosis difficult. Routine diagnostic imaging modalities mainly consist of endoscopic examination, computed tomography (CT) scanning, and magnetic resonance imaging (MRI)[7-12]. However, doctors who admit patients with acute or subacute obscure gastrointestinal bleeding (OGIB) prefer to conduct emergency interventional digital subtraction angiography (DSA), whereas some routine exams are not able to identify the bleeding[13-15]. To our knowledge, few detailed reports on the use of interventional DSA for small bowel GISTs have been published to date.

In this study, 28 small bowel tumors in 25 consecutive patients undergoing interventional angiography were confirmed as GISTs based on histopathology after surgical resection. Patients' medical data and the interventional DSA procedures were retrospectively analyzed, and the aim of the present study was to evaluate the diagnostic efficacy and the application of emergency interventional angiography for small bowel GISTs in patients with bleeding at Sun Yat-sen Memorial Hospital (Guangzhou, China).

MATERIALS AND METHODS

Patient characteristics

From January 2006 to December 2013, 25 patients with acute or subacute OGIB as their main clinical manifestation were admitted to our department for emergency angiography examination to determine whether there were signs of active bleeding requiring further interventional treatment. We retrospectively studied these patients' medical data and found 28 histopathologically confirmed GIST of the small intestine according to the DSA findings and location. The main clinical characteristics of the 25 patients are shown in Table 1. Before the DSA exam, the causes of bleeding in all of the patients were unclear based on routine endoscopy (gastric and colorectal endoscopes) and the following imaging modalities: abdominal ultrasound (n = 21), abdominal X-ray films (n = 16), abdominal CT plain scan (n = 7), and contrast-enhanced CT and CT angiography (n = 3). In one patient, a lower small intestine ileus was suspected based on abdominal X-ray, and a hypervascular mass in another patient was observed in the upper jejunum based on contrast-enhanced CT. However, enhanced CT did not facilitate a definite diagnosis of the mass as either hemangioma, vascular malformation or a small intestinal tumor by the radiological resident on duty. The review of the medical records was carried out with approval by the ethics committee of our hospital.

Table 1 Clinical characteristics of 25 cases

Characteristic	*n* (%)
Age (yr)	
< 40	6 (24.0)
⩾ 40	19 (76.0)
Sex	
Male	7 (28.0)
Female	18 (72.0)
Hemoglobin, g/L	
> 90	5 (20.0)
60-90	15 (60.0)
< 60	5 (20.0)
Symptoms	
Tarry or bloody stool	25 (100)
Hematemesis	1 (4.0)
Abdominal pain	8 (32.0)
Intermittent melena	11 (44.0)
Duration, mo	
< 1	15 (60.0)
> 1	10 (40.0)
RBC transfusion, U	19 (76.0)
2-4	10 (40.0)
5-16	9 (36.0)

Interventional DSA examination and bleeding management

The patients underwent emergency abdominal DSA that was performed with an Integris V3000 Vascular X-ray System (Philips, Ann Arbor, MI, United States) using a nonionic contrast agent (Ultravist, 300 mg I/mL; Bayer Schering Pharma AG, Berlin-Wedding, Germany). A 5-F introducer sheath was inserted into the right femoral artery using the improved Seldinger's technique. The tip of a 5-F Yashiro catheter was inserted into the celiac trunk, and the superior and inferior mesenteric arteries. Each DSA series lasted 20 s with 2 photos taken per second and using a 15 mL contrast bolus injected with a 903300 D Power Injector (Liebel-Flarsheim, Cincinnati, OH, United States). We evaluated the DSA images to determine their

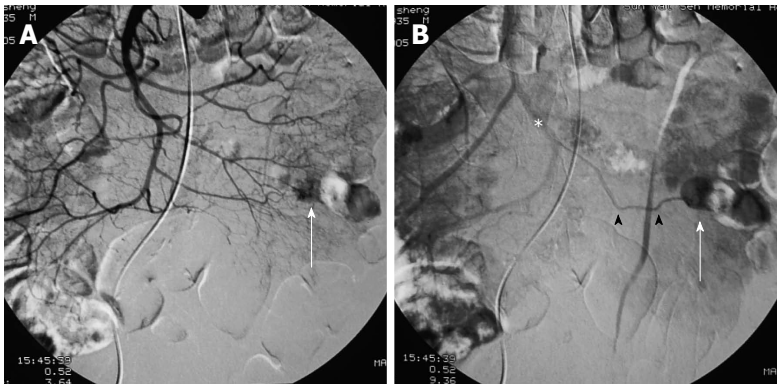

Figure 1 Small bowel gastrointestinal stromal tumor. A 70-year-old man with a 1.2 cm gastrointestinal stromal tumor of very low risk classification in the upper jejunum. The tumor (white arrows) was round, well-defined and homogeneous in the early arterial phase (A, B). The feeding arteries were not significantly enlarged, and the draining veins (B: black arrowheads) developed clearly and early, merging into the portal vein system (B, asterisk).

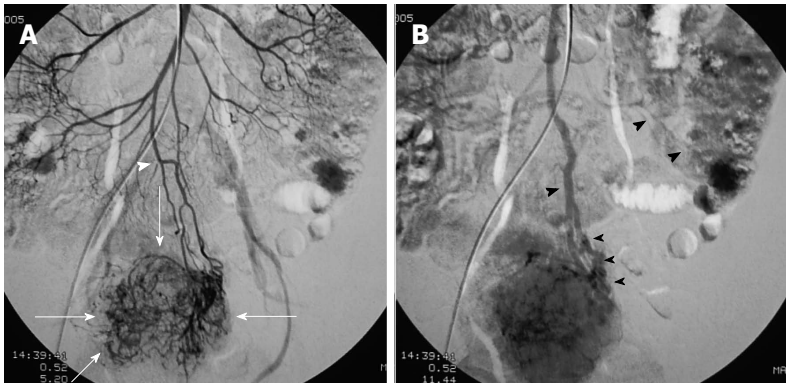

Figure 2 Larger and multiple small bowel gastrointestinal stromal tumors. A 52-year-old woman with 2 gastrointestinal stromal tumors in the middle jejunum. The tumors (white arrows) were 5.5 cm and 1.5 cm in diameter, respectively, and were also hypervascular and homogeneous in the early arterial phrase. The feeding artery of the large tumor (A: white arrowhead) was slightly thickened. The draining veins of the tumors (B: black arrowheads) were also clear. The pathological risk classification was high (large) and very low (small).

exact location, feeding artery, blood supply, number, boundary, draining veins and bleeding signs (extravasation of contrast agent). In the cases of tumor with severe blood loss, anemia or signs of obvious active bleeding, the tumor was embolized with gelfoam particles for hemostasis. The time of the DSA procedure was also recorded.

Surgery and pathological examination

All small bowel tumors underwent surgical resection following the guided site of interventional DSA. The operation records and histopathological exams included locations, capsule integrity, size, internal structure of the tumor, suspicious lymph nodes near the tumor, and pathologic diagnosis (histopathological and immunohistochemical findings). The diagnosis of the GISTs was based on the WHO classification system for soft-tissue tumors [Hamilton SR, Aaltonen LA. Pathology and Genetics. Tumors of the Digestive System. Lyon: IARC Press (2000)].

RESULTS

Patient characteristics

The clinical features of the 25 cases of small bowel GISTs are summarized in Table 1. The patient ages ranged from 34- to 70-year-old (average: 52 ± 12 years). Six patients were 30- to 39-year-old and 19 were over 40-year-old. The patients' chief clinical sign was acute or subacute OGIB with varying degrees of anemia. Forty-four percent of the patients had intermittent melena, and one patient also had acute hematemesis. The clinical history of GI bleeding ranged from 3 d to 10 years. Hemoglobin levels were 34 to 104 g/L (average: 68 ± 22 g/L) when patients presented to our hospital. Nineteen patients received a RBC transfusion of 2-16 U (average: 6.5 ± 2.0 U).

Appearance of small bowel GISTs on DSA and the effect of interventional management

First, abundant blood vessels, appearing as hypervascular tumors, were observed from the early arterial phase to the venous phase in masses of various sizes (Figure 1, Figure 2, Figure 3, Figure 4). The tumors were homogenously well-defined (smooth or mild lobular) or enhanced without filling defects (necrosis), arteriovenous fistulae/malformations or pseudoaneurysms. Second, the feeding artery was not obviously enlarged (Figure 1A) or was only slightly enlarged (Figures 2A and 3) in relation to tumor size. The feeding artery divided into numerous small coiled or hair-like branches into the tumor (Figures 1A,

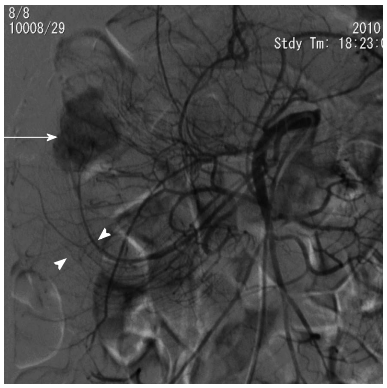

Figure 3 Small bowel gastrointestinal stromal tumor case combined with intussusception. A 34-year-old woman with tarry stool and abdominal pain. Digital subtraction angiography showed a 2.5 cm well-defined hypervascular tumor (white arrow) in the middle jejunum. The left branches of the superior mesenteric artery shifted to the upper right and was arm-shaped (white arrowheads; intussusception proved by surgery) around the tumor. The pathological risk of this patient was low.

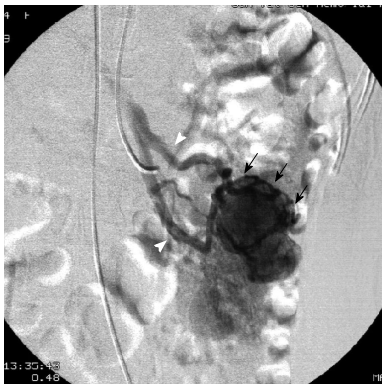

Figure 4 Small bowel gastrointestinal stromal tumor case with active bleeding. A 35-year-old woman with a 3.2 cm gastrointestinal stromal tumor in the middle jejunum. The hemoglobin was 34 g/L. Digital subtraction angiography showed many draining veins (arrows) on the tumor merging into the portal vein system (arrowheads) without signs of active bleeding. The tumor was slightly lobular and was low risk based on the pathological classification.

Table 2 Surgical and pathological findings of 28 tumors

Finding	*n* (%)
Location	
Jejunum	20 (71.4)
Ileum	8 (28.6)
Size (cm)	
≤ 2.0	6 (21.4)
2.1-5.0	20 (71.4)
≥ 5.1	2 (7.1)
Manifestations	
Ulceration	8 (28.6)
Erosion	10 (35.7)
Others	10 (35.7)
Risk of aggressive behavior	
Very low	6 (21.5)
Low	19 (67.9)
Moderate	2 (7.1)
High	1 (3.5)

2A and 3). Third, there was usually only one tumor (Figures 1, 3 and 4); multiple tumors were observed in only a few cases (Figure 2). No abnormal staining of lymph nodes near the tumor was observed (Figures 1B, 2B and 4). Fourth, obvious reticular and thickened draining veins were found in tumors, and the larger tumor, the thicker the draining veins (Figures 2B and 4). The draining veins developed early and merged into the portal vein system (Figures 1B, 2B and 4). These findings were more evident on super-selective angiography (Figure 4). Fifth, signs of active bleeding (extravasation of contrast agent) were not obvious. Although patients had a symptom of acute GI bleeding, no extravasation of contrast agent was detected (Figure 4). Finally, there were special DSA features when intussusception and other complications coexisted (Figure 3).

There were a total of 28 tumors detected by interventional DSA in these patients, with 22 cases involving single tumors and 3 cases involving multiple tumors (2 tumors each). Twenty of the GISTs were located in the jejunum and 8 in the ileum. Eight tumors in 7 patients with a large amount of blood loss were treated with transcatheter arterial embolization (TAE) with gelfoam particles during the DSA procedure.

The time per interventional procedure ranged from 22 to 45 min (average: 29 ± 5 min). All patients tolerated the interventional DSA and management well and did not develop any complications related to the procedure.

Surgical and pathological findings

All of the tumors detected on DSA were completely resected and shown to be GISTs of the small intestine based on pathological tests and immunohistochemical analysis. OGIB was resolved immediately after the procedure. In one patient, a tumor was found inside the small intestinal intussusception and correlated with the preoperative DSA findings. The exact location and number of tumors were consistent with the DSA results. The maximum diameter of the resected tumors ranged from 1.2 to 5.5 cm (average: 3.05 ± 1.25 cm) (Table 2). Eight of the tumors had associated ulceration, erosion was observed in 10 tumors, and hyperemia/edema was observed in 10 tumors on the intra-luminal side.

Based on the pathological and immunohistochemical evaluations, for example, hematoxylin-eosin staining and CD34-positivity indicated that abundant blood vessels in various diameters proved that the small bowel GISTs were hypervascular tumors (Figure 5 and Figure 6). The tumors were classified based on their various risks of aggressive behavior as shown in Table 2.

Reasons for interventional DSA

Of all the patients undergoing DSA, the most important reason was acute or subacute OGIB with low hemoglobin levels; the second most important reason was due to the physicians' clinical experience regarding the use of DSA in the detection of OGIB and the interventional treatment of the source of GI bleeding, such as arteriovenous malformations, artery rupture, and some GI tumors.

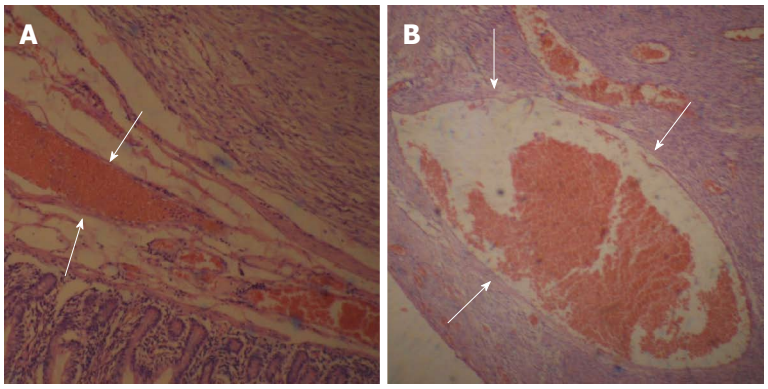

Figure 5 Representative findings from pathological examination of small bowel gastrointestinal stromal tumor. Two microscopic fields (A, B) of a hematoxylin-eosin stained gastrointestinal stromal tumor (GIST) shows a rich variety of tumor vessels filled with red blood cells (white arrows) (magnification × 40).

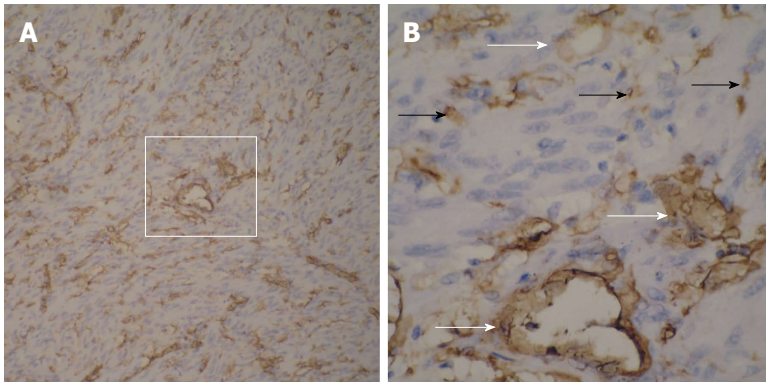

Figure 6 Representative findings from immunohistochemical examination of small bowel gastrointestinal stromal tumor. A: CD34-positive cells, as well as numerous blood vessels of various diameters (magnification × 100), are shown; B: The magnified imaging (A, white box) in which the CD34-positive tumor cells (B, black arrows) and vascular endothelial cells (B, white arrows) are clearly evident (magnification × 400).

DISCUSSION

For small bowel GISTs, early clinical manifestations are often absent or nonspecific, and mainly depend on tumor size and location. For the 25 patients in the present study, acute or subacute OGIB was the main indication for DSA, which was confirmed with subsequent surgical and pathological results. RBC transfusion, which was performed in the majority of the patients (19/25, 76.0%), was another indication for interventional treatment. Therefore, when conventional endoscopic and imaging examination fails to locate the lesions in patients with bleeding in a timely manner, emergency DSA may be a useful option. Other characteristics of the patients with small bowel GISTs, such as age, sex and abdominal pain, were similar to previous reports in the literature[2,5,6,13,16].

This study showed that there were some common DSA characteristics in small bowel GISTs. Despite the different tumor sizes, DSA can clearly reveal their exact site, range or number, blood supply and complications. Of the 28 small bowel tumors in our study, tumors measuring 2 cm or less (6/28, 21.4%) were also well-defined and exhibited obvious draining veins on the tumor surface with homogenous enhancement. Compared with the findings of Fang *et al*[17], which showed that draining veins were found only in 27.2% (3/11) of these tumors, we think that the different appearances of the draining veins are mainly based on tumor location, size, and classification. Although CT scans[2,9-11,13,17] also focus on the detection of feeding arteries and tumor blood supply, little attention has been paid to the draining veins of GISTs. In this study, in addition to the clear depiction of feeding arteries, numerous draining veins on tumors also appeared clearly in the early phase of the DSA procedures. The draining veins of the tumors merged into the portal venous system. Additionally, the draining veins were enlarged in proportion to the size of the tumor. We think that this feature may be used as one of the angiographic criterion for small intestinal GISTs and can be used to detect and localize smaller GISTs (especially $\leqslant$ 2 cm in our study). In addition, two reasons that the small bowel GISTs were relatively smaller compared with previous reports[1,10,11,14] include tumor bleeding and the timely use of the DSA procedure. Furthermore, to our knowledge, this article presents the largest DSA series of small bowel GISTs to date[13,17-20]. Based on our DSA findings, it is not difficult to detect and make a preoperative diagnosis and to locate small bowel GISTs.

Abdominal CT and MRI play an important role in the diagnosis of small intestinal tumors, especially larger size tumors, and facilitate the evaluation of their extent, fixed tumor structures, staging and the detection of abdomi-

nal metastasis[4,6,9-13,21]. Therefore, CT is often the initial imaging modality used to evaluate patients who present with non-specific abdominal symptoms or a palpable abdominal mass. Smaller GISTs typically appear as well-defined soft-tissue and low-density masses that are relatively homogeneous on enhanced CT. When the tumors are larger (usually, > 5 cm), they are often heterogeneous because of necrosis, hemorrhage, and myxoid degeneration. However, the appearance of GISTs on enhanced CT varies depending on the size, location and aggressiveness of the tumor. Wu *et al*[21] reported a 100 sample series of small bowel GISTs and found that the sensitivity of the detection of enhanced CT was 91%. However, other studies showed that the CT detection rate was low for tumors with intraluminal growth and for smaller sized tumors (< 35 mm)[22,23].

Capsule endoscopy (CE) and double-balloon enteroscopy (DBE) have completely changed the approach and launched a new era for clinical management of small bowel diseases[7,8,22,23]. Some studies suggested that these two modalities are diagnostically superior to other routine procedures, such as push enteroscopy, abdominal CT and small bowel angiography, in detecting small bowel lesions[24].

Compared with the diagnostic approaches mentioned above, interventional DSA is helpful and timely for detecting small bowel GISTs in patients with bleeding due to several advantages. Unlike the abdominal CT/MRI, CE or DBE procedures, DSA is more convenient and timely and patients do not need rigorous preoperative preparation[7-10,12,24]. In addition, the procedure time was not too long. Furthermore, the procedure is reasonably sensitive for detecting small bowel GISTs. Finally, if signs of active bleeding are observed during the DSA procedure, interventional radiologists can treat the bleeding. Our results are consistent with previous studies that showed that interventional radiological procedures in the detection of bleeding GISTs of the small bowel were superior to other diagnostic approaches[18].

In this study, the pathological and CD34-positive results showed that GISTs were hypervascular tumors that were free of cystic lesions. This finding may be related to the appearance of some small bowel GISTs on DSA and may indicate one reason for bleeding. However, it is not very clear why some patients exhibited the symptom of intermittent melena.

In general, this study has some limitations. First, the sample of tumors was too small for further analysis, including the number and size (> 5 cm) of the tumors. Second, although acute or subacute bleeding was the main indication for DSA, we found no obvious signs of extravasation of contrast agent, which indicates bleeding. Darnell *et al*[13] reported that one of three performed DSAs indicated signs of active bleeding. A possible explanation was a temporary resolution of bleeding because of the use of hemostasis and other factors before DSA. This result also explained the history of intermittent bleeding in some patients with GISTs in our study group (11/25, 44.0%). However, based on our experience and knowledge and due to the safety of surgical laparotomy, we performed TAE using gelfoam particles in 7 patients (8 tumors) with severe anemia due to actively bleeding tumors. Third, all tumors detected with DSA were treated with emergency surgery instead of preoperative arterial embolization *via* catheterization. For most surgeons, biopsy or embolization may increase an unconfirmed risk of rupture (causing peritoneal seeding) and intestinal ischemia[25].

In summary, our study showed that small bowel GISTs had some characteristic DSA features. Emergency interventional DSA was well tolerated in our patients with GI bleeding. We suggest that interventional DSA may be an ideal imaging selection in patients with small bowel GISTs that are unidentified by other methods and may be useful in making the diagnosis of the small bowel GISTs. We believe that interventional DSA is both a useful imaging option for locating and diagnosing of small bowel GISTs in patients with bleeding as well as an effective treatment modality.

COMMENTS

Background

Small bowel gastrointestinal stromal tumors (GISTs) are rare and are often non-specific or asymptomatic. Because of their deep location and more aggressive biobehavior, by the time these tumors are detected, they are usually larger and may be associated with abdominal masses or metastases. In some small bowel GIST patients with gastrointestinal bleeding and intermittent melena, treatment is delayed for various reasons, such as a resolution of bleeding after medical treatment, negative results from routine endoscopy and imaging analysis, and small tumor size. Furthermore, there have been a few reports on the detailed appearance of these lesions on digital subtraction angiography (DSA) in previous studies. This article demonstrates many new DSA findings in 28 small bowel GISTs in 25 patients.

Research frontiers

There are few studies that have focused on the appearance of small bowel GISTs on DSA. In this study, the authors demonstrate new DSA findings and the association between the DSA findings and pathological characteristics.

Innovations and breakthroughs

This is the first study with such a large sample of small bowel GISTs undergoing the DSA procedure. The authors focused on both the arterial phase and venous phase appearances, especially of the draining veins, which may be a very important imaging characteristic for the detection of smaller lesions. For patients with a large amount of blood loss, radiologists could perform interventional therapy to resolve bleeding during the DSA procedure. Therefore, interventional DSA is an effective theranostic approach.

Applications

The study provides another safe and effective theranostic modality for managing patients with small bowel bleeding, while other imaging examinations are not able to detect these bleeds.

Peer review

This is a retrospective study that shows the DSA features of small bowel GISTs. The authors concluded that because of some characteristic DSA features, emergency interventional DSA is both a useful imaging selection in locating and diagnosing of small bowel GISTs in patients with bleeding, as well as an effective treatment modality. This is a really fine paper. My opinion is that the authors did a great job.

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RETROSPECTIVE STUDY

Small sphincterotomy combined with endoscopic papillary large balloon dilation *vs* sphincterotomy alone for removal of common bile duct stones

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Abstract

AIM: To evaluate the efficacy and safety of endoscopic papillary large diameter balloon dilation (EPLBD) following limited endoscopic sphincterotomy (EST) and EST alone for removal of large common bile duct (CBD) stones.

METHODS: We retrospectively compared EST + EPLBD (group A, n = 64) with EST alone (group B, n = 89) for the treatment of large or multiple bile duct stones. The success rate of stone clearance, procedure-related complications and incidents, frequency of mechanical lithotripsy use, and recurrent stones were recorded.

RESULTS: There was no statistically significant difference between the two groups regarding periampullary diverticula (35.9% *vs* 34.8%, $P > 0.05$), pre-cut sphincterotomy (6.3% *vs* 6.7%, $P > 0.05$), size (12.1 ± 2.0 mm *vs* 12.9 ± 2.6 mm, $P > 0.05$) and number (2.2 ± 1.9 *vs* 2.4 ± 2.1, $P > 0.05$) of stones or the diameters of CBD (15.1 ± 3.3 mm *vs* 15.4 ± 3.6 mm, $P > 0.05$). The rates of overall stone removal and stone removal in the first session were not significantly different between the two groups [62/64 (96.9%) *vs* 84/89 (94.4%), $P > 0.05$; and 58/64 (90.6%) *vs* 79/89 (88.8%), $P > 0.05$, respectively]. The rates of post-endoscopic retrograde cholangiopancreatography pancreatitis and hyperamylasemia were not significantly different between the two groups [3/64 (4.7%) *vs* 4/89 (4.5%), $P > 0.05$; 7/64 (10.9%) *vs* 9/89 (10.1%), $P > 0.05$, respectively]. There were no cases of perforation, acute cholangitis, or cholecystitis in the two groups. The rate of bleeding and the recurrence of CBD stones were significantly lower in group A than in group B [1/64 (1.6%) *vs* 5/89 (5.6%), $P < 0.05$; 1/64 (1.6%) *vs* 6/89 (6.7%), $P < 0.05$, respectively].

CONCLUSION: EST + EPLBD is an effective and safe endoscopic approach for removing large or multiple CBD stones.

Key words: Choledocholithiasis; Endoscopic retrograde cholangiopancreatography; Endoscopic papillary balloon dilation; Endoscopic sphincterotomy

Core tip: Endoscopic papillary large diameter balloon dilation (EPLBD) after limited endoscopic sphincterotomy (EST) is an effective and safe endoscopic approach to remove large or multiple common bile duct stones. Compared with EST alone, the rate of bleeding and recurrence of CBD stones were significantly lower in the EST + EPLBD group (1.6% *vs* 5.6%, $P < 0.05$; 1.6% *vs* 6.7%, $P < 0.05$, respectively). While the rates of overall stone removal and stone removal in the first session (96.9% *vs* 94.4%, $P > 0.05$; 90.6% *vs* 88.8%, $P > 0.05$,

respectively) and the rates of post-endoscopic retrograde cholangiopancreatography pancreatitis and hyperamylasemia were not significantly different between the two groups (4.7% *vs* 4.5%, $P > 0.05$; 10.9% *vs* 10.1%, $P > 0.05$, respectively).

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INTRODUCTION

Although endoscopic sphincterotomy (EST) has been widely accepted as the standard therapy for removing common bile duct (CBD) stones, this procedure is associated with serious complications such as hemorrhage, pancreatitis, perforation, and recurrent infection of the bile duct caused by permanent functional loss of the sphincter of Oddi[1].

Endoscopic papillary balloon dilation (EPBD) of the biliary sphincter was introduced as an alternative to EST, especially for patients with small or moderate CBD stones[2]. By using the wire-guided method, EPBD could be easily performed. Because EPBD does not involve cutting the biliary sphincter, it possesses the advantages of preserving papillary sphincter function and reducing the chance of hemorrhage and perforation[3,4]. However, this procedure is associated with a high risk of pancreatitis[5] and with more frequent application of mechanical lithotripsy[6-8].

To overcome these disadvantages, endoscopic papillary large diameter balloon dilation (EPLBD) after limited EST was introduced for the removal of large (≥ 10 mm) or multiple bile duct stones[9-22]. This method combines the advantages of EST and EPBD by increasing the efficacy of stone extraction while minimizing complications of EST and EPBD when used alone[9,21].

This retrospective study aimed to evaluate the efficacy and safety of EPLBD after limited EST compared with EST alone for the removal of large (≥ 10 mm) or multiple CBD stones.

MATERIALS AND METHODS

Patients

A total of 153 patients with large (≥ 10 mm) or multiple CBD stones treated from January 2009 to December 2012 were retrospectively reviewed. Patients were excluded if they had a history of EST, a surgical history involving the gastrointestinal tract, co-existing bile leakage or choledochoduodenal fistula, bleeding tendency, intrahepatic stone diseases, or concomitant pancreatic or biliary malignant disorders. The patients were divided into two groups according to the order of the procedure. Sixty-four patients underwent EST + EPLBD (group A, from September 2011 to December 2012), and 89 patients underwent EST alone (group B, from January 2009 to September 2011). The study was approved by the Institutional Review Board of our hospital.

Methods

Prior to endoscopic retrograde cholangiopancreatography (ERCP), blood samples were obtained for a complete blood count, liver-function tests (bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase) before and the morning after the procedure, coagulation profiles, and serum amylase before and after the procedure (4 h and 24 h, respectively).

Written Informed Consent was obtained from all of the patients or from their relatives before the procedure. Local anesthesia of the pharynx was obtained using tetracaine. The patients were sedated with diazepam (5 mg) supplemented by an intramuscular injection of 50 mg of meperidine. To halt duodenal peristalsis, 20 mg of butyl scopolamine bromide was administered intramuscularly immediately prior to the start of ERCP. ERCP was performed by experienced endoscopists at a single center using side-viewing endoscopes (TJF-240; Olympus Optical Corporation, Tokyo, Japan).

After selective cannulation of the CBD using a triple lumen sphincterotome (Papillotome, ENDO-FLEX GmbH, Germany) with a guide wire (Hydra Jagwire 0.035 inch, Boston Scientific Microvasive. Cork, Ireland), a diagnostic cholangiogram was obtained, and the stone size and number were documented (Figure 1A). If cannulation of the CBD was not possible, a needle-knife sphincterotomy was performed to gain access. In group A, limited EST was performed (Figure 2A) for easy insertion of the balloon catheter and control of the direction of the balloon dilation during EPBD; then, a CRE wire-guided balloon catheter (5.5 cm in length, 1.0-1.2 cm/1.2-1.5 cm in diameter) (Boston Scientific Microvasive, Cork, Ireland) was passed over the guide wire and was positioned at the center of the balloon across the ampullary orifice. The balloon was gradually inflated with diluted contrast material to 12-15 mm at a pressure of 3-8 ATM, depending on the stone size and the diameter of the CBD (Figure 2B) as measured by cholangiography. The sphincter was adequately dilated when the waist of the balloon had disappeared completely (Figure 1B). The fully inflated balloon was maintained in its position for 2 min and then deflated and removed (Figure 2C). Following EPLBD, the stones were retrieved using a Dormia basket (Web™ extraction basket, Wilson-Cook Medical Inc. Winston-Salem, NC, United States) and/or a retrieval balloon catheter (Extractor Three Lumen Retrieval Balloon, Boston Scientific Microvasive. Cork, Ireland) (Figure 2D). When the stones were too difficult to remove intact, mechanical lithotripsy (BML-4Q; Olympus Optical, Tokyo, Japan) was performed to fragment the

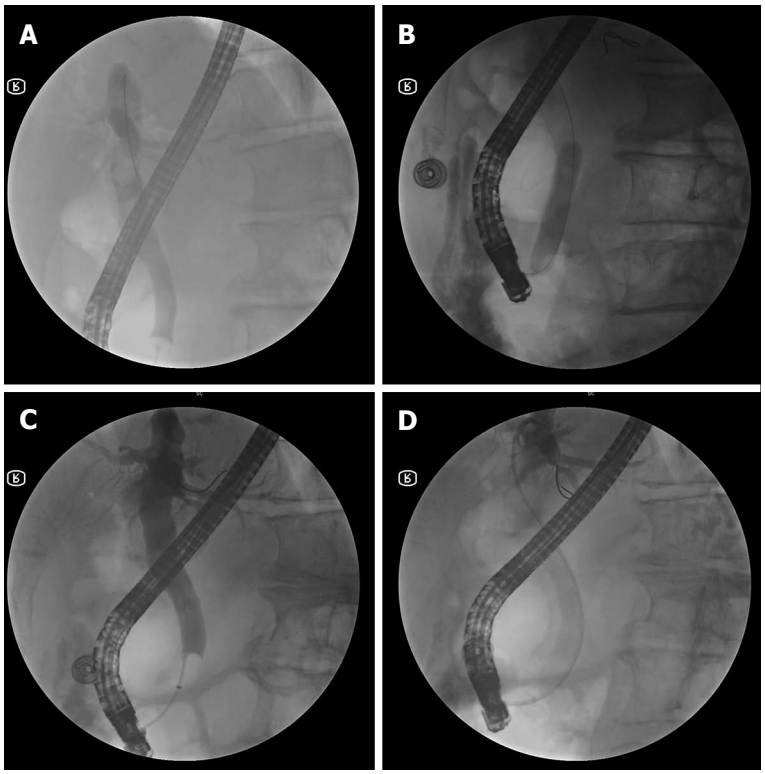

Figure 1 Fluoroscopic view of large-balloon dilatation following limited sphincterotomy. A: Cholangiogram demonstrating two large stones within the dilated bile duct; B: A large balloon inflated across the papilla over the guidewire; C: The cholangiogram following complete stone removal showed no residual filling defect in the bile duct; D: The placement of a nasobiliary drainage tube.

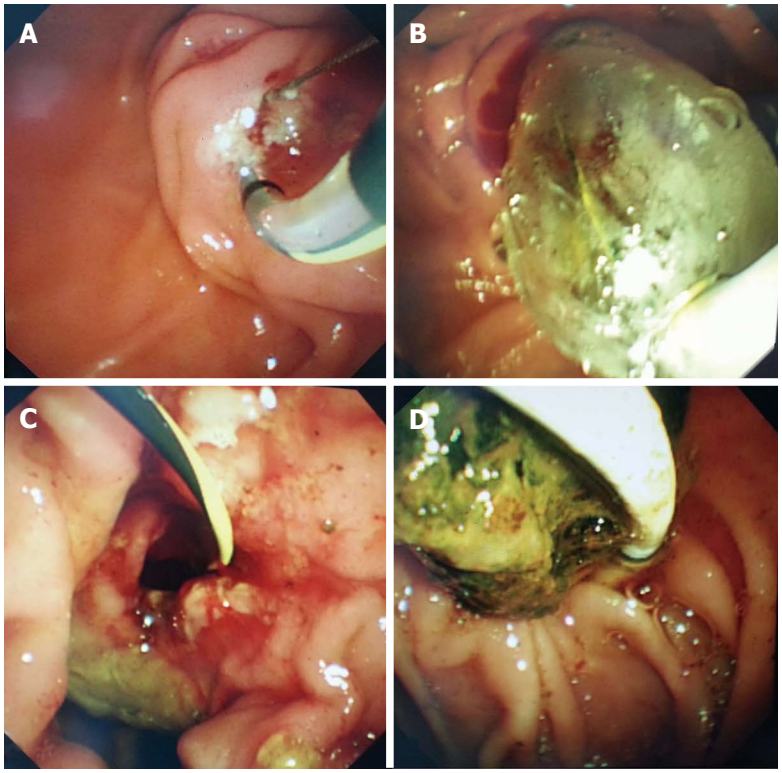

Figure 2 Endoscopic view of large-balloon dilation following limited sphincterotomy. A: Endoscopic small sphincterotomy; B: A large balloon inflated across the papilla; C: Markedly dilated papilla following large-balloon dilation; D: A large stone extracted using a retrieval balloon catheter through the dilated papilla.

Table 1 Baseline characteristics of the patients

Variable	Group A (n = 64)	Group B (n = 89)	P value
Mean age (yr)	68.5 ± 10.9 (39-87)	67.3 ± 9.8 (42-82)	> 0.05
Sex (male/female)	33:31	48:41	> 0.05
CBD stones			
Mean diameter of stones (mm)	12.1 ± 2.0 (10-25)	12.9 ± 2.6 (10-27)	> 0.05
Number of stones (1/2/≥ 3)	34/11/19	47/15/27	> 0.05
Mean diameter of CBD (mm)	15.1 ± 3.3 (10-26)	15.4 ± 3.6 (10-28)	> 0.05
Periampullary diverticula	23 (35.9%)	31 (34.8%)	> 0.05

stones prior to extraction from the bile duct, and a nasal biliary drainage tube (nasobil.Sonde, ENDO-FLEX GmbH, Germany) was placed to prevent cholangitis (Figure 1D). In group B, EST was performed with a pull-type sphincterotome as the standard method. Following EST, the stones were removed in the same manner as in group A. Complete stone removal was verified either by the final cholangiogram (Figure 1C) or by the follow-up cholangiogram obtained 3 d after the initial procedure through a nasobiliary drainage tube. If remnant stones were found, a second ERCP procedure with or without repeated EPLBD was performed for the retrieval of bile duct stones.

The outcomes measured were the number of therapeutic ERCP procedures required for complete stone removal, the frequency of use of mechanical lithotripsy, associated complications, including bleeding, pancreatitis, perforation during and after ERCP, and the recurrence of bile duct stones within one year. Post-ERCP pancreatitis was defined as persistent abdominal pain for more than 24 h associated with a serum amylase level of more than three times the upper limit of normal. Hyperamylasemia was defined as a serum amylase level exceeding three times the normal upper limit without any abdominal pain. Post-ERCP bleeding was classified as major or minor based on the amounts of hemorrhage. Major bleeding was defined as severe hemorrhage necessitating transfusion or interventions, and minor bleeding was defined as self-limited or endoscopically controlled mild hemorrhage not requiring transfusion. Cholangitis was defined as a fever accompanied by leukocytosis and right upper quadrant pain after the procedure[1]. Clinical and endoscopic factors (*e.g.*, periampullary diverticula) were retrospectively evaluated.

Statistical analysis

The data analyses were performed using the Statistical SPSS 10.0 software (Chicago, IL, United States). Categorical parameters were compared using the chi-square test or Fisher's exact test, and continuous variables were compared using the Student's *t*-test. All of the measurements in this study are expressed as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

The demographic characteristics of the 153 patients (81 men, 72 women; age range from 39 to 87 years) are presented in Table 1. The incidence of periampullary diverticula (PAD) was 35.3% (54/153). There was no statistically significant difference between the two groups in terms of the age and gender. The mean stone size in the 153 patients was 12.6 ± 2.4 mm (range, 10-27 mm), and the mean bile duct diameter was 15.2 ± 3.4 mm (range, 10-28 mm). There was no statistically significant difference between the two groups in terms of PAD (35.9% *vs* 34.8%, $P > 0.05$), pre-cut sphincterotomy (6.3% *vs* 6.7%, $P > 0.05$), size (12.1 ± 2.0 mm *vs* 12.9 ± 2.6 mm, $P > 0.05$) or number (2.2 ± 1.9 *vs* 2.4 ± 2.1, $P > 0.05$) of stones, or diameters of CBD (15.1 ± 3.3 mm *vs* 15.4 ± 3.6 mm, $P > 0.05$).

Of the 153 patients, stone removal was completed in 95.4% (146/153). The rates of overall stone removal and stone removal in the first session were not significantly different between the two groups [62/64 (96.9%) *vs* 84/89 (94.4%), $P > 0.05$, and 58/64 (90.6%) *vs* 79/89 (88.8%), $P > 0.05$, respectively]. The patients in group A required less mechanical lithotripsy compared with those in group B [3/64 (4.7%) *vs* 7/89 (7.9%), $P < 0.05$] (Table 2).

The procedure-related complications are listed in Table 3. The rates of post-ERCP pancreatitis and hyperamylasemia were not significantly different between the two groups [3/64 (4.7%) *vs* 4/89 (4.5%), $P > 0.05$; 7/64 (10.9%) *vs* 9/89 (10.1%), $P > 0.05$, respectively]. All of the cases of pancreatitis were mild, and they were treated conservatively. There were no perforations or cases of acute cholangitis or cholecystitis in the two groups. The rate of bleeding was significantly lower in group A than in group B [1/64 (1.6%) *vs* 5/89 (5.6%), $P < 0.05$]. There were 2 cases of major bleeding in group B; these patients later died from multi-organ failure. Regarding long-term complications, the recurrence of CBD stones was significantly higher in group B compared with group A [1/64 (1.6%) *vs* 6/89 (6.7%), $P < 0.05$].

DISCUSSION

EST, which was first introduced by Classen *et al*[21], remains the standard therapy for the treatment of CBD stones. Although EST has been proven to be safe in many studies, there are several complications, including pancreatitis (5.4%), hemorrhage (2.0%), perforation (0.3%), cholangitis (1.0%), cholecystitis (0.5%), and pro-

Table 2 Comparison of outcomes between the two groups *n* (%)

	Group A (*n* = 64)	Group B (*n* = 89)	*P* value
Precutting with needle knife	4 (6.3)	6 (6.7)	> 0.05
Mechanical lithotripsy	3 (4.7)	7 (7.9)	< 0.05
Overall stone removal	62 (96.9)	84 (94.4)	> 0.05
Complete stone removal in 1st session	58 (90.6)	79 (88.8)	> 0.05
Complete stone removal in 2nd session	4 (6.3)	5 (5.6)	> 0.05

Table 3 Comparison of complications between the two groups *n* (%)

	Group A (*n* = 64)	Group B (*n* = 89)	*P* value
Pancreatitis	3 (4.7)	4 (4.5)	> 0.05
Hyperamylasemia	7 (10.9)	9 (10.1)	> 0.05
Bleeding	1 (1.6)	5 (5.6)	< 0.05
Minor bleeding	1 (1.6)	3 (3.3)	
Major bleeding	0	2 (2.2)	
Mortality	0	2 (2.2)	
Perforation	0	0	
Acute cholangitis and cholecystitis	0	0	
Recurrence of CBD stones	1 (1.6)	6 (6.7)	< 0.05

cedure-related death (0.4%)[2].

EPBD has become an alternative to EST for the treatment of CBD stones. EPBD has several advantages over EST. First, EPBD results in less trauma to the ampullary sphincter. Second, EPBD might preserve the function of the biliary sphincter[2], reducing late complications such as the recurrence of biliary stones[23,24]. Third, EPBD has the advantage of less bleeding and is safer for patients with bleeding tendency. Finally, EPBD is recommended for patients with abnormal anatomy, such as periampullary diverticula and Billroth Ⅱ gastrojejunostomy, in which the margin for cutting is limited or the appropriate cutting direction is not clear[25]. However, a meta-analysis demonstrated that post-ERCP pancreatitis occurred more commonly in the EPBD group than in the EST group[8]. The balloon dilation of the sphincter of Oddi might cause spasm, compression and edema of the distal pancreatic duct, which could result in the restriction of pancreatic juice flow and the occurrence of pancreatitis[26]. Another disadvantage of conventional EPBD is that it is difficult to remove larger stones because the biliary opening is not enlarged to the same degree as with EST[6,7]; therefore, the application of EPBD is restricted to patients with small stones less than 10 mm in diameter[9].

EPLBD combined with limited EST, which was first proposed to facilitate the removal of large or multiple bile duct stones, has been proven safe and effective in patients with large bile duct stones[27]. EPLBD combined with limited EST enlarged the biliary orifice enough to remove multiple or larger bile duct stones, resulting in an increased success rate of stone removal[28,29] and in decreased use of mechanical lithotripsy[9,12,13,30-33]. In our study, compared with EST alone, the efficacy of stone removal was similar in EPBLD following limited EST. The rates of overall stone removal and stone removal in the first session were not significantly different between the two groups (96.9% *vs* 94.4%, $P > 0.05$, 90.6% *vs* 88.8%, $P > 0.05$, respectively). Periampullary diverticula, which are known to be associated with an increased frequency of pancreatobiliary diseases, could influence endoscopic outcomes because the ampullary area in patients with periampullary diverticula is composed of thin mucosa without sphincter muscle[34], which increases the potential risks of perforation and bleeding. In this case, mechanical lithotripsy is a necessary technique for removing large stones. However, the combination of EPLBD with limited EST provided spacious opening of the bile duct, reducing the need for mechanical lithotripsy (4.7% *vs* 7.9%, $P < 0.05$) in our study, which is consistent with previous reports[31]. For patients with difficult stones that are not suitable for extraction at the first attempt, the temporary placement of a stent might be an alternative method, and the plastic stents are able to fragment large CBD stones[35,36].

Pancreatitis is one of the most feared post-ERCP complications and occurs in 5%-19.8% of patients after EPBD[26]. Because EST guides the orientation of the dilating balloon towards the CBD and prevents pressure overload on the main pancreatic duct, the combined EPLBD with limited EST significantly decreased the risk of post-ERCP pancreatitis[37-39]. Moreover, the large balloon dilation results in a large opening of the bile duct, preventing accidental cannulation of the pancreatic duct in the subsequent stone extraction. To decrease the incidence of post-ERCP pancreatitis, cannulating the CBD selectively when performing the ERCP is important[29], and we used a sphincterotome with a guide wire instead of a catheter to avoid injecting contrast medium into the pancreatic duct. In our study, there were 7 patients who developed mild post-ERCP pancreatitis, including 3 cases in group A and 4 cases in group B. The patients recovered after conservative treatment in less than 72 h. Severe pancreatitis did not occur. There were 16 patients who developed post-ERCP

hyperamylasemia, including 7 cases in group A and 9 cases in group B. The elevated serum amylase level also normalized within 72 h after the procedure and did not affect the clinical course of the patients. There was no statistically significant difference between the two groups regarding post-ERCP pancreatitis and hyperamylasemia.

Regarding the risk of hemorrhage, we determined that limited EST prior to EPBD with a large balloon could reduce procedure-related hemorrhage. In our study, bleeding occurred less frequently in group A than in group B (1.6% *vs* 5.6%, $P < 0.05$). There were 2 cases of major bleeding in group B, and these patients later died from multi-organ failure. The other 4 cases had minor bleeding that was stopped by the administration of hemostatic agents. Limited EST and effective compression by a balloon are effective methods for the prevention of hemorrhage. Therefore, the combination of EST with EPLBD could be recommend for the removal of bile duct stones in patients who require anticoagulation[21]. Although there were some reports that EPLBD following limited EST resulted in a higher rate of bleeding, we attributed those results to the moderate degree of EST.

Another fatal complication during ERCP is perforation of the duodenum. However, during the ballooning after limited EST, the endoscopist could observe the dilation status of the ampulla using a sideview endoscope and fluoroscopy. Therefore, the risk of duodenal perforation during EST + EPLBD is lower than during EST alone, and the technique of EST + EPLBD is typically recommended in patients with periampullary diverticula. In our study, there were no cases of perforation in either group. To minimize the risk of perforation, the size of the dilated balloon should not exceed the size of the CBD.

Previous reports show that procedure-related acute cholangitis developed more often in the EST group than in the EPBD group. This result might be explained by the loss of sphincter function after EST, which enables bacterial colonization from the intestine into the biliary system[40]. In our study, there were no cases of acute cholangitis in either group, which could be attributed to application of endoscopic nasobiliary drainage.

The recurrence of stones and chronic biliary inflammation are long-term complications after bile duct stone extraction, especially in patients who undergo a large sphincterotomy. Mechanical lithotripsy might be another risk factor for stone recurrence because remnant stone fragments after lithotripsy could act as nidi for stone recurrence[31]. In our study, there were 7 cases of CBD stone recurrence; group A had 1 case, and group B had 6 cases. The results showed that EPLBD combined with limited EST decreased the recurrence of CBD stones compared with EST alone (1.6% *vs* 6.7%, $P < 0.05$). This decrease could be attributed to the preservation of the sphincter of Oddi, which prevents the chronic reflux of duodenal contents and bacteria into the biliary tree and to the lower frequency of mechanical lithotripsy. There were no cases of chronic biliary inflammation in either group; this might have been because of the short follow-up time.

EPLBD with limited EST is an effective and safe endoscopic approach for removing large or multiple CBD stones. However, this was a retrospective study, and the decision to perform EST alone or EPLBD with limited EST was made on an individual basis at the time of each examination. Further large randomized prospective case-controlled studies might be needed to confirm the efficacy and safety of EPLBD plus limited EST.

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COMMENTS

Background

Endoscopic sphincterotomy (EST) has been widely accepted as the standard therapy for removing common bile duct (CBD) stones; however, this procedure is associated with serious complications such as hemorrhage, pancreatitis, perforation, and recurrent infection of the bile duct. Endoscopic papillary balloon dilation of the biliary sphincter (EPBD) was introduced for patients with small or moderate CBD stones. This procedure has the advantages of preserving papillary sphincter function and reducing the chance of hemorrhage and perforation. However, it is associated with a high risk of pancreatitis and with more frequent application of mechanical lithotripsy.

Research frontiers

Recently, endoscopic papillary large diameter balloon dilation after limited endoscopic sphincterotomy (EST + EPLBD) was introduced for removing large (≥ 10 mm) or multiple bile duct stones. This method combines the advantages of EST and EPLBD by increasing the efficacy of stone extraction while minimizing complications of EST and EPBD when used alone.

Innovations and breakthroughs

The study showed that compared with EST alone, the patients in the EST + EPLBD group had lower rates of bleeding and recurrences of CBD stones, whereas the rates of overall stone removal and stone removal in the first session and the rates of post-ERCP pancreatitis and hyperamylasemia were not significantly different between the two groups. EST + EPLBD is a good alternative to conventional endoscopic sphincterotomy for the removal of large common bile duct stones. However, a larger study is required to clarify the advantages and disadvantages of this treatment.

Applications

Endoscopic papillary large diameter balloon dilation following limited sphincterotomy is effective and safe. An improved understanding of the advantages and disadvantages of the treatment for the removal of common biliary duct stones allows clinicians to make appropriate choices for patients.

Terminology

EST + EPLBD is defined as endoscopic papillary large diameter balloon dilation after limited endoscopic sphincterotomy. The balloon is positioned across the orifice of the ampulla and then is gradually inflated to an appropriate size.

Peer review

This paper compares the curative effect and safety of EST and EST + EPLBD for the treatment of bile duct stones. The authors concluded that EST + EPLBD is a good alternative to conventional endoscopic sphincterotomy for the removal of large or multiple common bile duct stones. This result provides valuable information for other researchers.

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RETROSPECTIVE STUDY

Effect of bilateral supraclavicular postoperative radiotherapy in middle and lower thoracic esophageal carcinoma

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Abstract

AIM: To evaluate whether postoperative radiotherapy is an alternative to neck lymph node surgery and if it provides a survival benefit for those receiving two-field, chest and abdomen, lymphadenectomy.

METHODS: A total of 530 cases with middle and lower thoracic esophageal carcinoma in our hospital from January 2008 to April 2009 were selected and analyzed, of which 219 cases received right chest, upper abdominal incision Ivor-Lewis surgery and simultaneously underwent mediastinal and abdominal two-field lymphadenectomy. If regional lymph node metastasis occurred within the recurrent laryngeal nerve, the patients would receive bilateral supraclavicular radiotherapy (DT = 5000cGy) to be adopted at postoperative 4-5 wk (Group A) or cervical lymphadenectomy at postoperative 3-4 wk (Group B). If there were no regional lymph node metastases within the recurrent laryngeal nerve, the patients only underwent two-field, chest and abdomen, lymphadenectomy (Group C).

RESULTS: In 219 cases who underwent two-field lymphadenectomy, 91 cases were diagnosed with regional lymph node metastasis within the recurrent laryngeal nerve. Of them, 48 cases received cervical radiotherapy, and 43 cases underwent staging lymphadenectomy; 128 patients were not given the follow-up treatment of cervical radiotherapy because there was no regional lymph node metastasis within the recurrent laryngeal nerve. Five-year survival rates in group A and B were 47% and 50%, respectively, with no statistical difference between them, and the rate in group C was 58%.

CONCLUSION: For patients with middle and lower thoracic esophageal carcinoma combined with lymph node metastasis within the recurrent laryngeal nerve, cervical radiotherapy can be a substitute for surgery and provide benefit.

Key words: Middle and lower thoracic esophageal carcinoma; Lymph node metastasis; Bilateral supraclavicular postoperative radiotherapy

Core tip: Three-field lymphadenectomy is currently a focus in esophageal surgery. According to the patterns of lymph node metastasis, understanding of the lymph node status in the middle and lower recurrent laryngeal nerve region may predict the status of cervical lymph nodes. In patients who have received thoracic and abdominal lymphadenectomy, if lymph node metastasis of recurrent laryngeal nerve is indicated it will be especially important to subsequently treat cervical lymph nodes. Additional cervical lymphadenectomy is

always performed clinically in this case. In this article, we discuss whether cervical surgical procedures can be replaced by radiotherapy.

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INTRODUCTION

Tumor cell metastasis *via* the lymphatic pathway is a major spread pathway of esophageal carcinoma. Many studies have demonstrated that esophageal carcinoma occurs more frequently at lymph nodes of the bilateral recurrent laryngeal nerve[1-3]. Especially for middle and lower thoracic esophageal carcinoma, once lymph node metastasis occurs in this region, it often indicates the presence of lymph node metastasis[4-7]. For those patients, three-field lymphadenectomy (including neck, chest, and abdominal lymph nodes) is often adopted. Three-field lymphadenectomy expands the scope of the operation, with benefits for the survival of patients[8-10]. However, it combines with an ensuing increase in surgical trauma and postoperative complications[11-17]. Cervical lymph node metastasis is not common[1], and not all patients will benefit from the three-field lymphadenectomy. On the other hand, surgery has its own limitations, and it can only reach the level of radical resection from the naked eye. Many studies have reported that patients who underwent three-field lymphadenectomy still have risks of recurrent cervical lymph node after surgery. Radiation therapy, as with surgery, can kill localized tumor cells, and has the advantage of being non-invasive compared with surgery. The question is whether or not we can adopt bilateral supraclavicular radiotherapy to replace lymphadenectomy, and achieve control effects on cervical lymph nodes. Based on this idea, we designed the supplemental treatment modality of cervical radiotherapy after two-field lymphadenectomy.

MATERIALS AND METHODS

After preoperative examination, patients who had a definitive diagnosis of esophageal squamous cell carcinoma, with the location of the tumor in the middle and lower thoracic cavity, expected to achieve R0 resection at surgery, with no evidence of distant metastasis were included. Exclusion criteria: (1) the presence of preoperative palpation or ultrasound or positron emission tomography computed tomography (PET-CT) indicated that there were obvious cervical lymph nodes, highly suspicious transfers, or after pathological examination, cervical lymph node metastasis was confirmed (such patients need surgery before radiotherapy and chemotherapy); (2) preoperative diagnosis by CT or endoscopic ultrasound (EUS) of T4 (such patients tend to have lower rates of surgical resection, surgery is required before radiotherapy and chemotherapy); (3) patients with severe disease (severe cirrhosis, diabetes or heart and lung complications); and (4) patients with a history of previous gastric resection surgery (usually not as a substitute for the esophageal tube after surgery).

Patients with lower thoracic and cardia cancer, or confirmed adenocarcinoma through preoperative histological examination, were also excluded (such as specific pathological types, and if there was a possibility that accumulation at lower esophageal gastric region was present).

A total of 530 cases with middle and lower thoracic esophageal carcinoma in our hospital from January 2008 to April 2009 were selected and analyzed, of which 219 cases receiving right chest, upper abdominal incision Ivor-Lewis surgery, and simultaneously undergoing mediastinal and abdominal two-field lymphadenectomy, met the above criteria. Of 219 cases, 91 cases were diagnosed with regional lymph node metastasis within the recurrent laryngeal nerve. Among these, 48 cases received cervical radiotherapy at postoperative 4-5 wk (Group A), 43 cases underwent second cervical lymphadenectomy at postoperative 3 wk (Group B); 128 cases of postoperative recurrent laryngeal nerve pathology without lymph node metastasis received only two-field lymphadenectomy.

Surgical treatment

Abdominal surgical procedures: After abdominal incision, patients received abdominal surgery. Tissue was removed at a distance of $\geqslant$ 5 cm from the lower edge of the tumor, cardia and proximal stomach, where a gastric tube would be made. Simultaneously, abdominal lymph node resections were undergone (including the 16th set of pericardial lymph nodes, 17th set at drainage area of the left gastric artery, 18th set at the drainage area of common hepatic artery, 19th set at traveling area of splenic artery, 20th set of lymph nodes around the celiac artery) as shown in Figure 1.

Thoracic surgical procedures: Thoracic surgery was undergone through a posterolateral incision at the right side, dissociated almost from the entire thoracic esophagus; meanwhile, mediastinal lymph node regions were removed on the recurrent laryngeal nerve (2, 3, and 4 sets), as shown in Figure 2A and 2B, especially the cervicothoracic junction within the thoracic lymph entrance, and the middle and lower mediastinal lymph nodes (7, 8, 9, 10, and 16 sets), as shown in Figure 2C.

At the edge of the tumor lesions at $\geqslant$ 5 cm, the esophagus was cut, and a gastric tube was put into the thoracic cavity as a substitute. Through the esophageal bed, the stomach and top right pleural esophagus was anastomosed.

Postoperative radiotherapy

At postoperative 3-4 wk, the patients began to receive three-dimensional conformal radiotherapy at cervical and bilateral supraclavicular regions. Radiation fields included the bilateral supraclavicular region (upper boundary was the cricothyroid membrane level, lower bound-

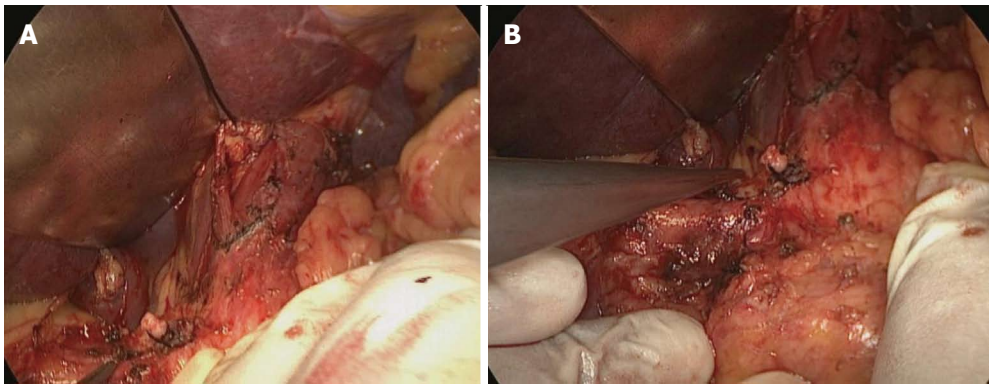

Figure 1 Abdominal surgical procedures. A: Bilateral diaphragmatic foot anatomy, focusing on clear phrenic lymph (16 set); B: Celiac artery anatomy, focusing on lymph node dissection of 17, 18, 19, 20 sets.

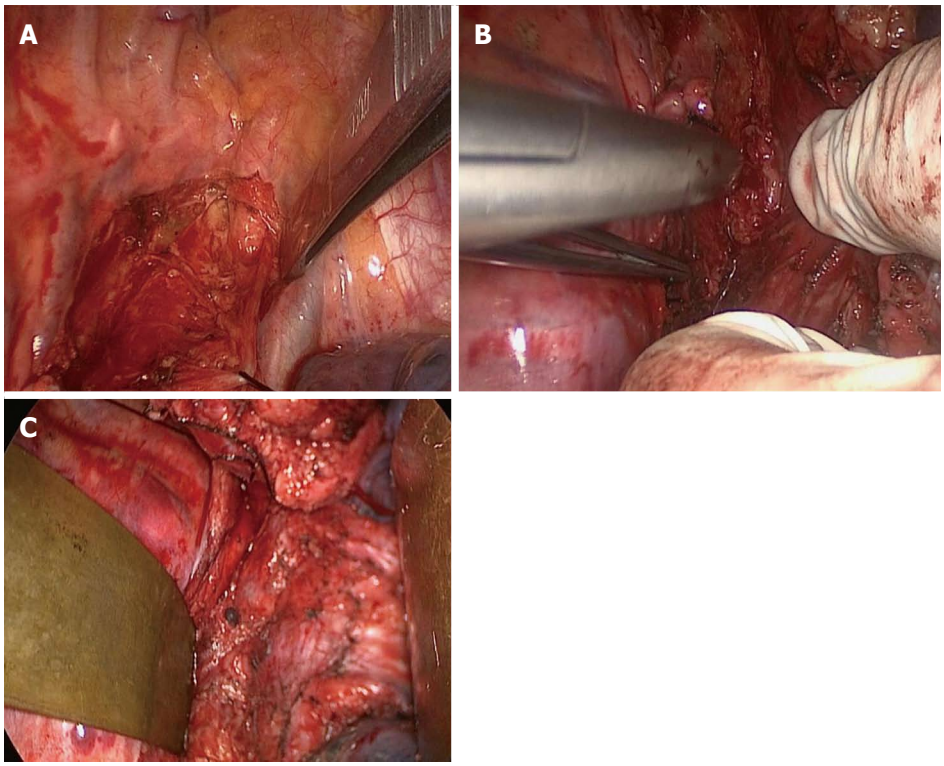

Figure 2 Thoracic surgical procedures. A and B: Mediastinal lymph node regions on the recurrent laryngeal nerve 2, 3, and 4 sets; C: Middle and lower mediastinal lymph nodes 7, 8, 9, 10, and 16 sets.

ary was the clavicular head level) as shown in Figure 3: 8 MV X-ray accelerator irradiation DT = 50GY/25 times, 2GY per time, 5 times/wk.

Statistical analysis

The data were analyzed using Mann Whitney's *U*-test and Fisher's exact test. Kaplan-Meir survival curves were used to analyze the subsistence data. The differences between groups were compared using log-rank test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Comparison of clinical features

The characteristics of the three groups are shown in Table 1. The UICC TNM classification (the 7th edition) was used for staging. Significant differences in patient age, gender and tumor location were not observed.

Complications comparison

Postoperative complications occurred in 4 cases (4/48,

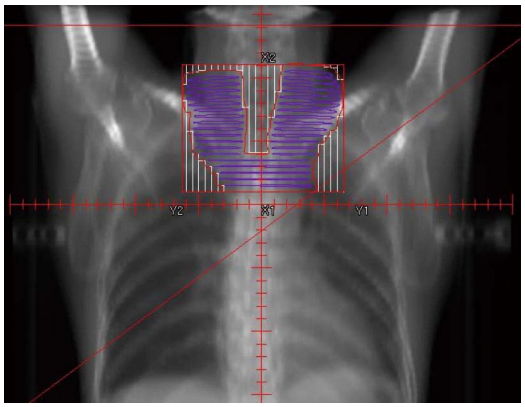

Figure 3 Radiation field included bilateral supraclavicular region.

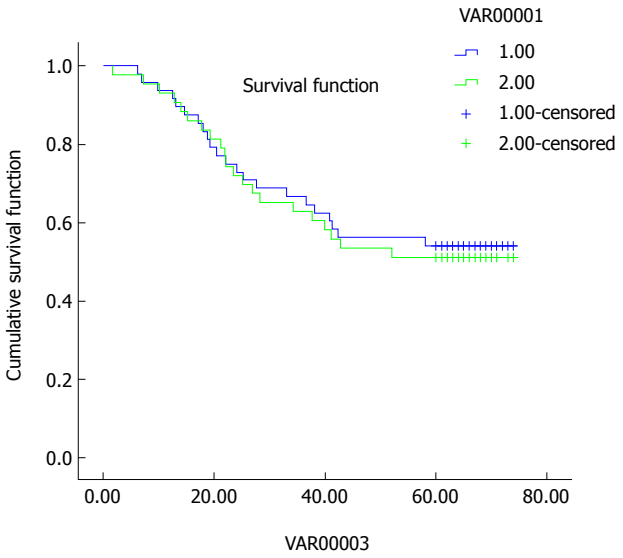

Figure 4 Cause-specific survival curves for groups A and B.

8.3%) in group A, and in 8 cases (8/43, 18.6%) in group B; 1 patient in group B died due to postoperative respiratory failure during hospitalization (Table 2).

Comparison of recurrence and metastasis

During the follow-up period, 60 cases had tumor recurrence or metastasis, among which were 18 cases of group A, 19 cases of group B and 23 cases of group C (Table 3). Two cases of group C had cervical lymph node metastasis. Lymph node metastasis was the most common in groups A and B; hematogenous metastasis was more common in group C (Table 3).

Comparison of survival

Cause-specific survival curves for groups A and B are shown in Figure 4. The 5-year survival rates of groups A and B were not significantly different, at 47% and 50%, respectively.

DISCUSSION

Surgery is the main treatment of esophageal carcinoma.

Table 1 Characteristics of the three groups

Parameters	Group A (*n* = 48)	Group B (*n* = 43)	Group C (*n* = 128)	*P*
Age (yr)	62.1 + 7.6	62.8 + 7.3	63.1 + 5.6	
Sex				
Male	47	43	125	NS
Female	1	0	3	
Tumor location, *n*				
Middle thoracic	30	27	73	NS
Lower thoracic	18	16	55	
PT, *n*				
T1a	4	3	9	NS
T1b	8	5	17	
T2	18	17	48	
T3	16	17	46	
T4a	2	1	8	
PN, *n*				
N0	0	0	128	
N1	23	19	0	NS
N2	16	16	0	
N3	9	8	0	
PG, *n*				
G1	31	28	61	NS
G2	15	13	59	
G3	2	2	8	

NS: Not significant.

Table 2 Postoperative complications, *n*

Complications	Group A	Group B	*P*
Anastomotic leak	1	1	NS
Recurrent laryngeal nerve palsy	1	2	NS
Respiratory failure	0	1	NS
Chylothorax	1	2	NS
Wound infection	1	2	NS
Hospital death	0	1	NS

Table 3 Recurrence and metastasis during follow-up period, *n*

Parameters	Group A	Group B	Group C
Cervical lymph nodes	1	1	2
Upper mediastinum (regional lymph nodes)	2	1	2
Middle and lower mediastinal lymph nodes	6	7	2
Abdominal lymph nodes	6	5	3
Hematogenous metastasis	3	5	14
Total	18	19	23

As complete a removal of lymph node metastasis as possible is needed to ensure surgical efficacy. According to the characteristics of lymphatic drainage of esophageal carcinoma[18], bilateral cervical, chest and abdominal mediastinal may be the transferred areas; in theory, the removal of all nodes at the above-mentioned three regions (called three-field lymphadenectomy) can achieve maximum efficacy. Among the many clinical reports, the survival period of patients receiving three-field lymphadenectomy are generally higher than that of patients receiving mediastinal and abdominal lymphadenectomy[8-10]. However, three-

field extended lymphadenectomy increases trauma and perioperative complications in patients[12-16]. Our study also found that patients who received three-field lymphadenectomy appeared to have a higher incidence of recurrent laryngeal nerve palsy and anastomotic leak compared with those who received two-field lymphadenectomy. Thus, it is controversial whether all patients should consistently receive three-field lymphadenectomy.

Some studies suggest that analyzing from the anatomy, regional lymph nodes of recurrent laryngeal nerve are a part of cervical lymph nodes. Thus, in the order of lymphatic drainage[19], lymph at the submucous layer of the esophagus is firstly drained to the recurrent laryngeal nerve, then to the neck[20,21]; regional lymph nodes of the recurrent laryngeal nerve are considered as the neck sentinel lymph nodes. In clinical practice, both Noguchi *et al*[22] and Noguchi[22] did pathological examinations of recurrent laryngeal nerve lymph nodes for patients who underwent IVOR-Lewis, and chose positive patients with recurrent laryngeal nerve lymph node metastasis to receive stepwise or contemporaneous neck lymph node dissection, while those without lymph node metastasis in this area received only two-field lymphadenectomy. There was no significant difference in clinical survival rate over the same period for lymphadenectomy patients. Li *et al*[23] found that, especially for patients with middle and lower thoracic esophageal carcinoma, the recurrent laryngeal nerve region might suggest lymph node status; therefore it is recommended to select the appropriate patients with cervical lymphadenectomy. Many studies support the idea that not all patients with esophageal carcinoma have lymph node metastasis present, and patients without lymph node metastasis cannot benefit from a three-field lymphadenectomy[1]. From this perspective, we did not give two-field lymphadenectomy for 128 patients without regional lymph node metastasis, and postoperative 5-year survival rate was 58%; therapeutic effect was achieved on an equal with similar literature. We believe that from the primary tumor resection perspective, Ivor-Lewis surgery can guarantee the thoroughness of middle and lower thoracic esophageal carcinoma resection. If three-field lymphadenectomy is not selected, it will make some patients have too much normal esophagus cut, stomach esophageal anastomosis will be forced at the neck, which only increases the pressure on surgery; it may bring more complications and affected the postoperative quality of life in patients.

In our study, only 91 cases with postoperative recurrent laryngeal nerve lymph nodes from 219 patients received two-field lymphadenectomy; these were considered to need follow-up treatment on cervical lymph nodes. The similarities of radiotherapy and surgery are that both of them can kill local tumor cells, but radiotherapy has the comparative advantage over surgery in that it is relatively non-invasive, and can reduce the patients' psychological and physical adverse effects. Thus, for patients receiving two-field lymphadenectomy, can radiation therapy be used to replace the operation when there is the possibility of cervical lymph node metastasis? Based on this idea, we had 91 cases of two-field lymphadenectomy, of whom 48 patients were treated with postoperative cervical radiotherapy and 43 cases were given cervical lymphadenectomy. The results showed there was no significant difference in cervical lymph node recurrence rate between the two groups, while the 5-year survival rate was not significantly different in comparison. In other words, with this model, it can still achieve the efficacy of lymph node dissection without additional surgery. Postoperative cervical radiotherapy can reduce the pressure on doctors, reduce surgical trauma to patients, avoid neck incision, and be more easily accepted mentally.

In conclusion, for middle and lower thoracic esophageal carcinoma patients, postoperative radiotherapy after bilateral supraclavicular lymph node surgery can avoid cervical lymph node surgery, and the clinical efficacy is the same as with three-field lymphadenectomy.

COMMENTS

Background

A surgical procedure is the main method for treatment of esophageal cancer, and usually includes three aspects: tumor resection, lymph node resection and digestive tract reconstruction. Regarding lymph node resection, the scope and eradication are key factors influencing the surgical efficiency and also the focus of clinical research. Due to the special anatomic and lymphatic draining characteristics, neck, chest and abdomen are possible locations of metastasis. Theoretically, resections of all the possible lymph nodes may maximize the eradication, which however causes increased trauma during surgery. Currently, resections of thoracic and abdominal lymph nodes are the consensus of scholars dedicated to esophageal surgery. Thus, how to choose the appropriate patients for cervical lymphadenectomy and whether some surgical procedures can be replaced by non-surgical methods are investigated in this study.

Research frontiers

Physicians have obtained favorable clinical outcomes in diagnosis of cervical lymph node metastasis by palpation and cervical ultrasound. Latest studies have found that according to the patterns of lymph node metastasis, lymph nodes in the recurrent laryngeal nerve region may serve as the outpost of cervical lymph nodes. Based on this, selective three-field lymphadenectomy may be performed to obtain better therapeutic efficacy.

Innovations and breakthroughs

In this study, the authors did not use techniques such as ultrasound, computed tomography, *etc*. to predict the lymph node status, but predicted cervical lymph node status according to the patterns of lymph node metastasis and pathological status of lymph nodes in the recurrent laryngeal nerve region. Based on this, selective subsequent treatment was performed, which avoided false positive results and micro-metastasis of technical methods. The obvious difference from previous studies is that they replaced traditional surgery with radiotherapy, which reduced the injuries of patients while relieving the mental pressure of both physicians and patients.

Applications

This study proved that it is feasible to selectively perform cervical lymphadenectomy according to the patterns of lymph node metastasis and the pathological status in the thoracic recurrent laryngeal nerve region. Therefore, surgical procedures might be replaced by regional radiotherapy. Furthermore, the regional recurrence rate and the 5-year survival rate were not significantly different from surgical procedures. Additionally, radiotherapy produces lighter injuries than surgery. Therefore, it is more easily accepted by patients and widely used clinically.

Terminology

Three-field lymphadenectomy: this is a surgical procedure with additional cervical lymph node resection based on two-field lymphadenectomy (thoracic and abdominal field). The scope of cervical lymphadenectomy involves bilateral supraclavicular lymph nodes, cervical para-esophageal lymph nodes and anterior cervical lymph nodes.

Peer review

The manuscript is very well written. For the patients with middle and lower thoracic esophageal carcinoma, once the regional lymph node metastasis occurs within the recurrent laryngeal nerve, it often indicates the presence of lymph node metastasis, which requires three-field lymphadenectomy. A total of 530 cases with middle and lower thoracic esophageal carcinoma are included. The results suggest that for patients with middle and lower thoracic esophageal carcinoma complicated with lymph node metastasis within the recurrent laryngeal nerve, cervical radiotherapy can substitute surgery and provide benefits.

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RETROSPECTIVE STUDY

Cost-effectiveness analysis of colon cancer treatments from MOSIAC and No. 16968 trials

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Abstract

AIM: To compare XELOX and FOLFOX4 as colon cancer adjuvant chemotherapy based on MOSAIC and No. 16968 trails from Chinese cost-effectiveness perspective.

METHODS: A decision-analytic Markov model was developed to compare the FOLFOX4 and XELOX regimens based MOSAIC and No. 16968 trial. Five states were included in our Markov model: well (state 1), minor toxicity (state 2), major toxicity (state 3), quitting adjuvant chemotherapy (state 4), and death due to adjuvant chemotherapy (state 5). Transitions among the 5 states were assumed to be Markovian. Costs were calculated from the perspective of the Chinese health-care payer. The utility data were taken from published studies. Sensitivity analyses were used to explore the impact of uncertainty factors in this cost-effectiveness analysis.

RESULTS: Total direct costs of FOLFOX4 and XELOX per patient were $19884.96 ± 4280.30 and $18113.25 ± 3122.20, respectively. The total fees related to adverse events per patient during the entire treatment were $204.75 ± 16.80 for the XELOX group, and $873.72 ± 27.60 for the FOLFOX4 group, and the costs for travel and absenteeism per patient were $18495.00 for the XELOX group and $21,352.68 for the FOLFOX4 group. The base-case analysis showed that FOLFOX4 was estimated to produce an additional 0.06 in quality adjusted life years (QALYs) at an additional cost of $3950.47 when compared to the XELOX regimen over the model time horizon. The cost per QALY gained was $8047.30 in the XELOX group, which was $900.98 less than in the FOLFOX4 group ($8948.28). The one way sensitivity analysis demonstrated that the utility for the well state and minor toxicity state greatly influenced the incremental cost-effectiveness ratio of FOLFOX4.

CONCLUSION: In term of cost-comparison, XELOX is expected to dominate FOLFOX4 regimes; Therefore, XELOX provides a more cost-effective adjuvant chemotherapy for colon cancer patients in China.

Key words: Cost-effectiveness; Adjuvant chemotherapy; Colon cancer; FOLFOX; XELOX

Core tip: Notably, patients with stage Ⅲ colon cancer are recommended to receive either XELOX or FOLFOX4 as adjuvant therapy. However, there has not been a

cost-effectiveness analysis of these two regimens. This study compared XELOX and FOLFOX4 as adjuvant chemotherapy for patients with colon cancer based on the MOSAIC and No. 16968 trails from a Chinese cost-effectiveness perspective. Our results demonstrated that XELOX was a more cost-effective treatment for adjuvant chemotherapy of colon cancer in China.

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INTRODUCTION

Colorectal cancer ranks third among the leading causes of cancer-associated death in both males and females of all ages worldwide after lung and breast cancer[1]. Nearly 70% colorectal cancers are located in the colon, and approximately 26% are in the stage Ⅲ when they were first diagnosed. What is even worse, it is estimated that about 50%-60% of patients will suffer a recurrence after radical resection[2].

Recently, many results of large clinical randomized controlled studies have showed that adjuvant chemotherapy after standard surgery and radiation for early stage colon cancer takes an active part in decreasing the risk of recurrence and extend overall survival[3]. An early pooled analysis has shown the efficacy of 5-fluorouracil/leucovorin (FU/LV) treatment with a significant reduction of the recurrence rate by 35%, and death rate by 22%, when compared with no treatment in patients with a curative resection of colon cancer[4]. Additionally, FU/LV has been shown to have the best efficacy among chemotherapy regimens, such as the FU and levamisole regimens[5]. This has led to FU/LV being the standard adjuvant chemotherapy for colon cancer until, that is, the MOSAIC trial found that FOLFOX4 (5-Fu/LV + OX) significantly increased disease-free survival (DFS) and overall survival (OS) when used as adjuvant treatment for stage Ⅱ or Ⅲ colon cancer[6,7].

Subsequently in 2011, the No. 16968 trial showed that compared with 5-FU/LV, XELOX led to significantly better 3-year DFS (66.5% *vs* 70.9%) and higher 5-year OS rate(74.2% *vs* 77.6%) in patients with stage Ⅲ colon cancer, respectively[8]. Therefore, based on the clinical practice guidelines of the 2011 National Comprehensive Cancer Network (NCCN), FOLFOX4 and XELOX were standard adjuvant treatments of colon cancer[9].

Several studies have consistently reported that compared with FU/LV, FOLFOX is a cost-effective adjuvant chemotherapy for patents with stage Ⅱ and Ⅲ colon cancer from the perspectives of United States, Canada and Japan[10-12]. Shiroiwa *et al*[13] suggested that XELOX, as first-line and second-line chemotherapy, was superior to FOLFOX4 in term of cost and effectiveness. Previously, we have indicated that XELOX is expect to outperform the FOLFOX4 regimen in China based on a simple cost analysis[14]. However, to the best of our knowledge, we do not know which regimen is more cost-effective. Hence, in this study we determined to find a more affordable adjuvant chemotherapy option between XELOX and FOLFOX4 for colon cancer patients based on data obtained from the MOSAIC and No. 16968 trials from a Chinese cost-effectiveness perspective.

MATERIALS AND METHODS

A Markov model was constructed to estimate the incremental cost-effectiveness of FOLFOX4 compared with XELOX as adjuvant treatments for patients who had stage Ⅱ or Ⅲ colon cancer in a Chinese health-care setting based on data obtained from the MOSAIC trial and No. 16968 trial. Our analysis was performed from a third-party health-care payer's perspective, which included both direct medical costs and indirect costs. Quality adjusted life year (QALY) gained, and an estimate of overall costs, were used to evaluate the incremental cost-effectiveness ratio (ICER) of both treatments.

XELOX and FOLFOX4 regimens

According to the No. 16968 trial, the XELOX regimen was a 3-wk cycle treatment for 8 cycles, including a 2-h intravenous infusion of oxaliplatin (130 mg/m^2) on day 1 and oral capecitabine (1000 mg/m^2) twice a day from day 1 to day 14[8]. FOLFOX4 was consisted of 2-h intravenous infusions of oxaliplatin(85 mg/m^2) and LV 200 mg/m^2 on day 1, followed by a bolus of 400 mg/m^2 of 5-FU and a 22-h infusion of 5-FU 600 mg/m^2 lasting 22 h every 14 d, for 12 cycles[6].

Groups of itemized expenses

In our base-case analysis, all expenses were separated into three categories: direct costs of chemotherapy (fees for anti-cancer drugs, hospitalization, venous access, experimental tests and pre-chemotherapy drugs, such as antiemetics and hepatoprotective drugs), costs of treatments related to adverse effects (for example, recombinant human granulocyte/macrophage colony-stimulating factor), and costs from a societal perspective (*i.e.,* travel fees and absenteeism cost). Direct chemotherapy costs and adverse effects related costs were analyzed from the perspective of healthcare providers, and travel costs and absenteeism costs were from societal perspective. Travel costs were based on taxi fare per kilometer in Sichuan, China. According to the median monthly salary in Sichuan, China (patient's salaries were not recorded in the medical records), equal costs were estimated for patients' hospital time. All the costs were translated into United States dollars. Other unrelated care fees were not calculated in the study.

Adjuvant chemotherapy transition model

Referring to the cost-effectiveness analysis of adjuvant

chemotherapy for colon cancer patients, 5 states were included in our Markov model (Figure 1) and states 1-5 were listed orderly as follows: well, minor toxicity, major toxicity, quitting adjuvant chemotherapy, and death due to adjuvant chemotherapy[15]. Transitions among the 5 states were assumed to be Markovian.

The first three states represented the toxicity reaction of patients received treatments according to the Common Toxicity Criteria of the National Cancer Institute, version 3, and the records of adverse events of the MOSAIC trial and No. 16968 trial, where "well" represented grade 0 toxicity, "minor toxicity" represented grade 1 and 2 toxicity, and "major toxicity" represented grade 3 and 4 toxicity, and severe or life-threatening adverse effects[16,17]. The cycle length of the Markov model was set to 1 mo for a total of 6 mo-specifically, the transitions occurred once a month for 6 times. At the start of the adjuvant chemotherapy, all patients began in the "well" state (free of cancer).

Pw-w, Pw-mi, Pw-ma, Pmi-w, Pmi-mi, Pmi-ma, Pma-w, Pma-mi, Pma-ma, Pma-q, and Pma-d were applied to denote the probabilities of transition of the model, in which suffix w represented the first state(well state), mi represented state of minor toxicity, ma represented state of major toxicity, q represented state of quitting the adjuvant chemotherapy, and d represented death state due to the adjuvant chemotherapy. Pw-mi represented the probability of the changing of patient in the well state in the current cycle to the minor toxicity state in the next cycle. Because the incidence of toxicity in our patients was consistent with the incidences found in several other clinical studies, the values for adverse events values reported in the MOSAIC trial and No. 16968 trial for FOLFOX4 and XELOX chemotherapy were applied in this study. A calibration method was used to estimate the exact values[15,18]. The parameter values are listed in Table 1.

Utility data for patients in adjuvant chemotherapy

Several related studies were examined to determine the utility values of the 5 states in the adjuvant chemotherapy[15,19]. The utility for well state patients was set to 0.84, which was the same for patients without adjuvant chemotherapy after surgery based on the study by Ramsey *et al*[19] and van Hout *et al*[20]. With regard to the utilities of minor toxicity and major toxicity, the means of utilities of patients with moderate or severe adverse events were calculated. The values are shown in Table 2.

Sensitivity analysis

One-way deterministic sensitivity analyses, a tornado diagram and threshold analysis were used to identify key model input parameters that could potentially influence the results over the low/high value, such as direct costs of chemotherapy, adverse related fees, societal costs, and utility scores. Based on the influence of the variables on the incremental net health benefit, a tornado diagram was applied. Willingness to pay was set to $17815.40, triple the per capita GDP of China according to the guidelines of World Health Organization for cost-effectiveness analysis[21,22].

RESULTS

Results of base-case analysis

No significant differences were identified for male/female ratio, age, depth of invasion and histology between the FOLFOX4 group and the XELOX group based on the MOSAIC trial and the No. 16968 trial (Table 3).

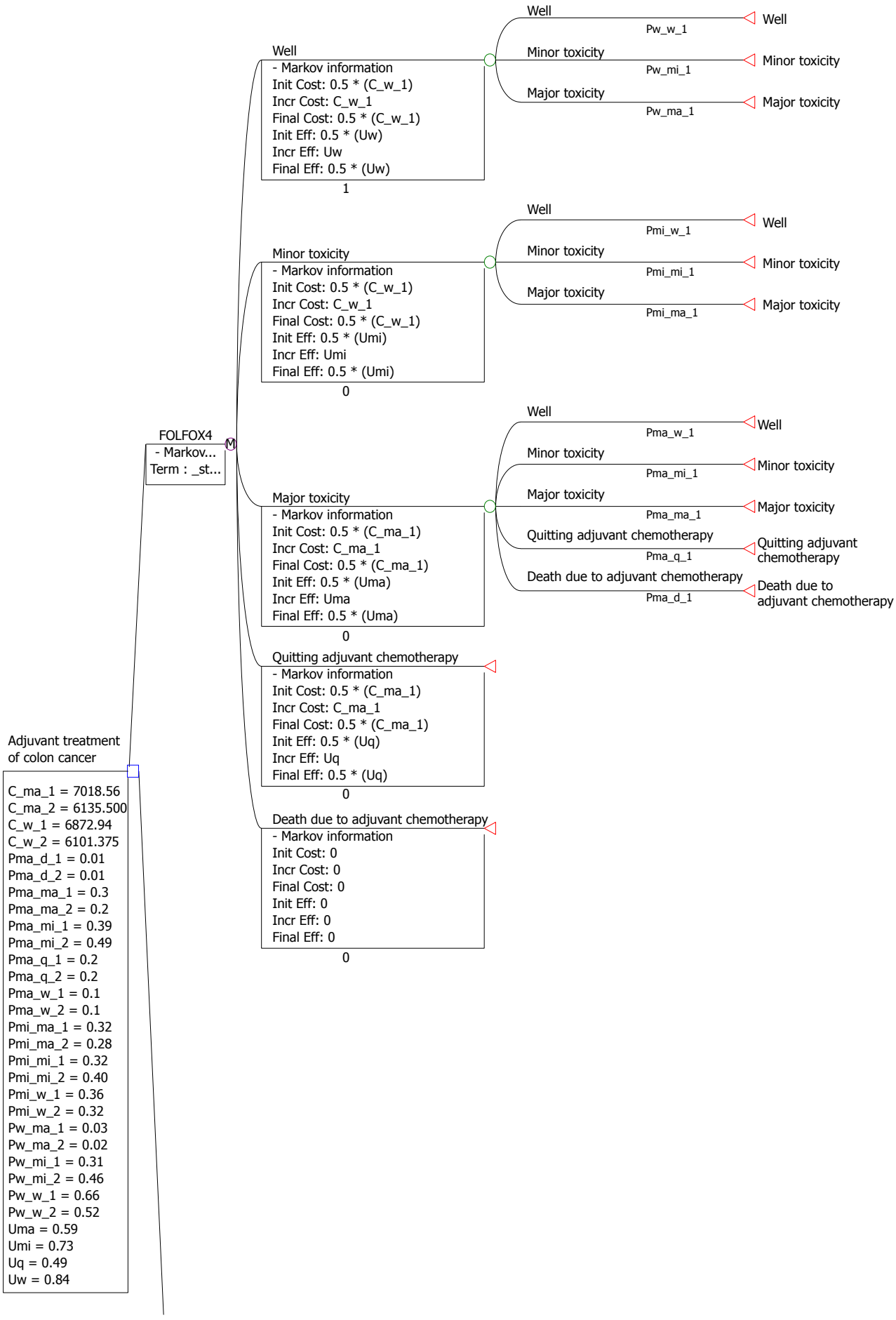
According to the cases in our study, the total direct costs of FOLFOX4 were 109.8% as high as those of XELOX per patient, which were $19884.96 ± 4280.30 and $18113.25 ± 3,122.20, respectively. Total fees related to adverse events per patient for the entire treatment were $204.75 ± 16.80 for the XELOX group, and $873.72 ± 27.60 for the FOLFOX4 group. For the costs from a societal perspective, specially, fees for travel and absenteeism, the average travel costs per cycle were set at $7.90 for taxi identically in two groups. Therefore, total travel costs would be $59.25 for XELOX per patient and $85.32 for FOLFOX4. Pay for one day was $15.70 according to the average monthly salary of $478 in Sichuan, China. As a result, the estimated costs for absenteeism for chemotherapy per patient were $508.68 in FOLFOX4 group and $117.75 in XELOX group, respectively. Hence, the total fees for travel and absenteeism per patient were $18495.00 for the XELOX group and $21352.68 for the FOLFOX4 group (Table 4).

Based on the data collected above, the cost for the well state was $6101.38 ± 520.37 in the XELOX group, and $6872.94 ± 713.38 in the FOLFOX4 group, which was identical to the minor toxicity state. The cost for the major toxicity state and quitting adjuvant chemotherapy state $6135.50 ± 523.17 in the XELOX group, and $7018.56 ± 717.98 in the FOLFOX4 group.

After running our Markov model for the 5 stages, the cumulative cost and cumulative effect were $30466.45 and 3.79 quality-adjusted life years for the XELOX group, and $34416.92 and 3.85 quality-adjusted life years for the FOLFOX4 group. Though the FOLFOX4 regimen was estimated to produce an additional 0.06 QALYs, the additional cost was significant ($3950.47) compared to the XELOX regimen over the model time horizon. The cost per QALY gained was $8047.30 in the XELOX group, which is $900.98 less than in the FOLFOX4 group ($8948.28). The results showed that XELOX was expected to dominate FOLFOX4 regimens; in other words, XELOX was a more cost-effective adjuvant treatment for colon cancer (Table 5).

Sensitivity analysis

The results of the sensitivity analysis for "cost for well state" (C_w_1 for FOLFOX4, C_w_2 for XELOX) and "cost for major toxicity state" (C_ma_1 for FOLFOX4, C_ma_2 for XELOX), and the "utility scores for 5 states" are shown in the tornado diagram (Figure 2).


Adjuvant treatment of colon cancer
C_ma_1 = 7018.56
C_ma_2 = 6135.500
C_w_1 = 6872.94
C_w_2 = 6101.375
Pma_d_1 = 0.01
Pma_d_2 = 0.01
Pma_ma_1 = 0.3
Pma_ma_2 = 0.2
Pma_mi_1 = 0.39
Pma_mi_2 = 0.49
Pma_q_1 = 0.2
Pma_q_2 = 0.2
Pma_w_1 = 0.1
Pma_w_2 = 0.1
Pmi_ma_1 = 0.32
Pmi_ma_2 = 0.28
Pmi_mi_1 = 0.32
Pmi_mi_2 = 0.40
Pmi_w_1 = 0.36
Pmi_w_2 = 0.32
Pw_ma_1 = 0.03
Pw_ma_2 = 0.02
Pw_mi_1 = 0.31
Pw_mi_2 = 0.46
Pw_w_1 = 0.66
Pw_w_2 = 0.52
Uma = 0.59
Umi = 0.73
Uq = 0.49
Uw = 0.84
FOLFOX4
- Markov...
Term : _st...
Well
- Markov information
Init Cost: 0.5 * (C_w_1)
Incr Cost: C_w_1
Final Cost: 0.5 * (C_w_1)
Init Eff: 0.5 * (Uw)
Incr Eff: Uw
Final Eff: 0.5 * (Uw)
1
Well
Pw_w_1
Well
Minor toxicity
Pw_mi_1
Minor toxicity
Major toxicity
Pw_ma_1
Major toxicity
Minor toxicity
- Markov information
Init Cost: 0.5 * (C_w_1)
Incr Cost: C_w_1
Final Cost: 0.5 * (C_w_1)
Init Eff: 0.5 * (Umi)
Incr Eff: Umi
Final Eff: 0.5 * (Umi)
0
Well
Pmi_w_1
Well
Minor toxicity
Pmi_mi_1
Minor toxicity
Major toxicity
Pmi_ma_1
Major toxicity
Major toxicity
- Markov information
Init Cost: 0.5 * (C_ma_1)
Incr Cost: C_ma_1
Final Cost: 0.5 * (C_ma_1)
Init Eff: 0.5 * (Uma)
Incr Eff: Uma
Final Eff: 0.5 * (Uma)
0
Well
Pma_w_1
Well
Minor toxicity
Pma_mi_1
Minor toxicity
Major toxicity
Pma_ma_1
Major toxicity
Quitting adjuvant chemotherapy
Pma_q_1
Quitting adjuvant chemotherapy
Death due to adjuvant chemotherapy
Pma_d_1
Death due to adjuvant chemotherapy
Quitting adjuvant chemotherapy
- Markov information
Init Cost: 0.5 * (C_ma_1)
Incr Cost: C_ma_1
Final Cost: 0.5 * (C_ma_1)
Init Eff: 0.5 * (Uq)
Incr Eff: Uq
Final Eff: 0.5 * (Uq)
0
Death due to adjuvant chemotherapy
- Markov information
Init Cost: 0
Incr Cost: 0
Final Cost: 0
Init Eff: 0
Incr Eff: 0
Final Eff: 0
0

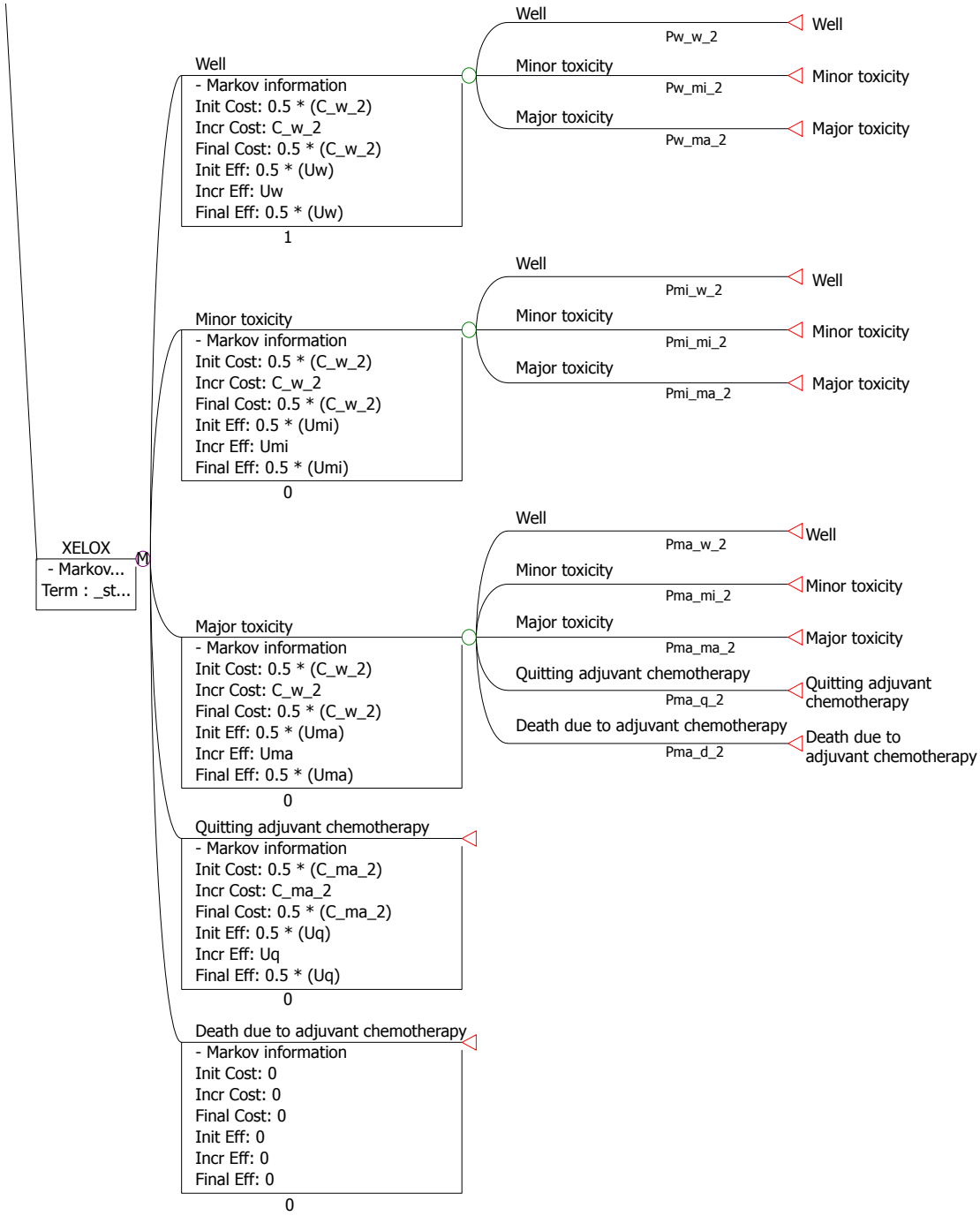


Figure 1 Markov model built for the cost-effectiveness analysis.

The results of one-way deterministic sensitivity analyses showed that the utility for the well state and the minor toxicity state greatly influenced the ICER of FOLFOX4. When the utility score of the minor toxicity state changed from 0.60 to 0.84, the ICER increased from $30242 per QALY gained to $5205921 per QALY gained, which was a highly significant increase. Among all the cost factors for the 5 states in the two groups, the cost for well state for the XELOX group (C_w_2) played a key role in our analysis, which varied from $5581.01 to $6621.75 and resulted in the ICER decreasing from $107342 per QALY gained to $23724 per QALY gained.

DISCUSSION

New adjuvant therapies for colon cancer have increased the overall survival, meanwhile patients' quality of life has been improved. However, a dramatic economic burden was produced with the widespread use of adjuvant treatments[13,21,23,24]. Indeed, the head-to-head comparisons trials of these different therapies are seldom, and a cost-effectiveness evaluation of the standard adjuvant chemotherapies in a health resource-limited setting is of critical importance to address the balance between health care costs and benefits. With indirect comparison and decision

Table 1 Parameters used for calculation

Parameters	Value		XELOX
	FOLFOX4	Data source	
Overall toxicity after adjuvant chemotherapy			
Percentage of major toxicity state Pma	50.9%	43.0%	Andréand others, 2004
Percentage of minor toxicity state Pmi	41.1%	55.0%	Hans-Joachim Schmoll and
Percentage of well state Pw	8.0%	2.0%	others, 2007
Percentage of death due to adjuvant chemotherapy Pd	0.5%	0.6%	
Percentage of quitting adjuvant chemotherapy Pq	25.3%	22.0%	
Probability of well state to well state Pw-w	0.66	0.52	Mehmet US Ayvaci and
Probability of well state to minor toxicity state Pw-mi	0.31	0.46	others, 2012
Probability of well state to major toxicity state Pw-ma	0.03	0.02	
Probability of minor toxicity state to well state Pmi-w	0.36	0.32	
Probability of minor toxicity state to minor toxicity state Pmi-mi	0.32	0.40	
Probability of minor toxicity state to major toxicity state Pmi-ma	0.32	0.28	
Probability of major toxicity state to well state Pma-w	0.10	0.10	
Probability of major toxicity state to minor toxicity state Pma-mi	0.39	0.49	
Probability of major toxicity state to major toxicity state Pma-ma	0.30	0.20	
Probability of major toxicity state to quitting adjuvant chemotherapy Pma-q	0.20	0.20	
Probability of major toxicity state to death due to adjuvant chemotherapy Pma-d	0.01	0.01	

Table 2 Utility data for patients in adjuvant chemotherapy

Parameters	Value	Data source
Well state	0.84	Mehmet US Ayvaci and
Minor toxicity state	0.73	others, 2012
Major toxicity state	0.59	
Quitting adjuvant chemotherapy	0.47	
Death due to adjuvant chemotherapy	0.00	

Table 3 Patients' baseline characteristics according to the MOSAIC and No. 16968 trials

	FOLFOX4 (n = 1123)	XELOX (n = 944)
Male, %	56.1	54
Age, yr	61	61
Depth of invasion, %		
T1-2	4.5	11
T3	76	74
T4	19	15
TX	0.5	< 1
Histology, %		
Differentiated	83.2	81
Poorly differentiated	12.6	15
Unknown	4.2	3

analysis modeling techniques, monetary costs and therapeutic efficacies of the adjuvant chemotherapy regimens FOLFOX4 and XELOX for patients with colon cancer were estimated over an adjuvant chemotherapy horizon in term of Chinese healthcare system based on MOSAIC trial and No. 16968 trial.

The XELOX group not only showed advantages in term of costs of hospitalization, and time and travel costs for the patient, but also in the cost of venous access. Based on these cost data, our results showed the whole cost of the XELOX regimen was 86.6% that of the FOLFOX4 regimen for an average patient, even though the gained quality-adjusted life years were approximately the same; 3.79 for XELOX and 3.84 for FOLFOX4. Though the FOLFOX4 regimen was estimated to produce an additional 0.06 QALYs, the additional cost was significant ($3950.47) compared to the XELOX regimen over the model time horizon. However, the cost was $8047.30/QALY in the XELOX group, and $8948.28/QALY in the FOLFOX4 group, which shows that the cost for the FOLFOX4 group was $900.98 greater. The results indicated that XELOX is worth considering as an alternative to the FOLFOX4 regimen and the results were consistent with the cost-effectiveness analysis in metastatic colorectal cancer[13].

In China, no consensus has been reached on the threshold of acceptable cost per QALY saved. A threshold range of £20000 to £30000 is used in the National Institute for Health and Clinical Excellence in the United Kingdom[25], whereas the United States often applies a threshold range of $50000 to $100000. Based on the guidelines of the World Health Organization (WHO) for cost-effectiveness analysis, the willingness to pay $17815.40, which is triple the per capita GDP of China, is an appropriate threshold[21]. Considering all the criteria above, the costs per QALY in the FOLFOX4 group and the XELOX group, as well as the ICER for FOLFOX4, were thought to be acceptable.

In our research, the rates of grade 3/4 adverse events related to FOLFOX4 and XELOX were obtained from the cross-trial comparison of the MOSAIC trial and No. 16968 trial, which showed that the incidences of diarrhea, stomatitis, nausea, vomiting, neurosensory, hand-foot syndrome (HFS), neutropenia and febrile neutropenia were 19% and 12%, 0.6% and 3%, 5% and 5%, 6% and 6%, 11% and 12%, 5% and 2%, 9% and 41%, 0.2% and 2% for FOLFOX4 and XELOX, respectively[7]. The differences were statistically significant for stomatitis, neutropenia and febrile neutropenia, resulting in higher adverse events-associated costs for FOLFOX4 regimen than that for XELOX regimen. Therefore, the cost for the major toxicity state was significantly higher in the

Table 4 Total costs for patients undergoing adjuvant chemotherapy

Parameters	FOLFOX4 ($)	XELOX ($)	Data source
Direct costs/mo	3314.16 ± 713.38	3018.88 ± 520.37	Xie *et al*[14] and others, 2013
Adverse event costs/mo	145.62 ± 4.6	34.13 ± 2.8	
Societal costs/mo	3558.78	3082.5	
Cost for well state (Cw1/Cw2)	6872.94 ± 713.38	6101.38 ± 520.37	
Cost for minor toxicity state (Cmi1/Cmi2)	Cw1	Cw2	
Cost for major toxicity state (Cma1/Cma2)	7018.56 ± 717.98	6135.50 ± 523.17	
Cost for quitting adjuvant chemotherapy (Cq1/Cq2)	Cma1	Cma2	

Table 5 Cost-effectiveness analysis of the base case

Parameters	FOLFOX4	XELOX
Whole cost	$34416.92	$30466.45
QALY	3.85	3.79
Cost/effect	$8948.28	$8047.30
ICER	$15016.33	-
Threshold (/QALY)	$17815.4	$17815.4

QALY: Quality adjusted life year; ICER: Incremental cost-effectiveness ratio.

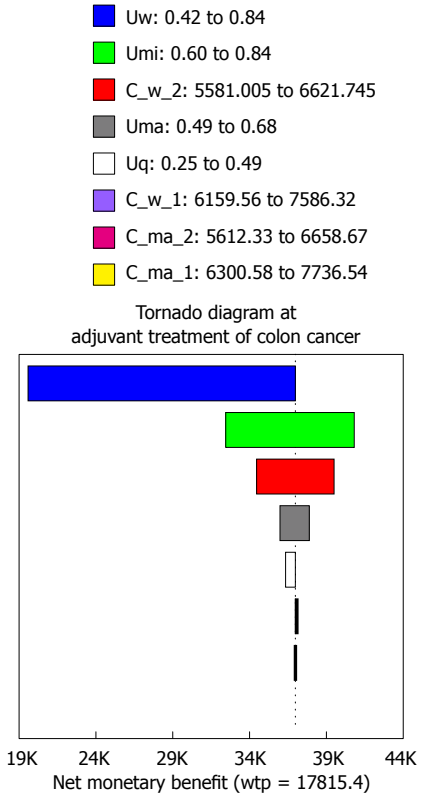


Figure 2 Tornado diagram summarizing the results of the one way sensitivity analysis.

FOLFOX4 group. However, from the results of one-way deterministic sensitivity analyses, it was not the cost for the major toxicity state (C_ma_2) but the cost for the well state for the XELOX group (C_w_2) that, which played a key role in our analysis-this varied from $5581.01 to $6621.75 and resulted in a reduction of ICER from $107342 per QALY gained to $23724 per QALY gained. Our results indicated that adverse effect-related costs might not be as important a factor to consider for optimal adjuvant chemotherapy.

To our surprise, the ICER of FOLFOX4 was greatly influenced by the utility scores for the well state and minor toxicity state. When the utility score of the minor toxicity state changed from 0.60 to 0.84, the ICER increased from $30242 per QALY gained to $5205921 per QALY gained. The reason for this may be that status of patients in adjuvant chemotherapy play a key role in estimating the QALY and evaluating the ICER[26].

Our research is partly based on patient-level data which were collected from the MOSAIC trial and the No. 16968 trial, and on the data collected from the West China Hospital, Sichuan University, China, which is the main limitation of our research. The potential heterogeneity of effectiveness and resource consumption between Asians and the international population may be another significant limitation in this research. Additionally, although we have analyzed the costs related to adverse events and societal costs, such as fees for travel and absenteeism, which are seldom included in other related cost-effectiveness analyses. Additionally, supportive care costs were not included in our analysis because that it is difficult to collect the detail from medical records. Finally, as we do not have direct head-to-head comparisons on the effectiveness of FOLFOX4 and XELOX for adjuvant treatment of patients with colon cancer in large randomized controlled trials, more detail should be studied to figure out the further cost-effectiveness of the two regimens.

Notably, it is the first study to compare cost-effectiveness option of adjuvant chemotherapy for colon cancer between FOLFOX4 and XELOX. We found that XELOX could achieve the maximum level of clinical benefit over other adjuvant treatments, which is a more affordable option in China. This result is worthy of consideration for both doctors and patients, and provides decision makers a more comprehensive view of treatment-related cost-effectiveness in clinical practice.

COMMENTS

Background

Based on the clinical practice guidelines of the 2011 National Comprehensive Cancer Network, FOLFOX4 and XELOX were standard adjuvant treatments of colon cancer. Several studies have consistently reported that compared with FU/LV, FOLFOX is a cost-effective adjuvant chemotherapy for patents with stage Ⅱ and Ⅲ colon cancer. However, which is a more affordable option of

adjuvant chemotherapy for colon cancer in the terms of cost-effectiveness analysis is still unknown.

Research frontiers

The base-case analysis showed that FOLFOX4 was estimated to produce an additional 0.06 in quality adjusted life years at an additional cost of $3950.47 when compared to the XELOX regimen over the model time horizon. The cost per QALY gained was $8047.30 in the XELOX group, which is $900.98 less than in the FOLFOX4 group ($8948.28). XELOX is expected to dominate FOLFOX4 regimens at a point view of cost-comparison point of view; in other words, XELOX was a more cost-effective treatment for adjuvant chemotherapy for patients with colon cancer in China.

Innovations and breakthroughs

It is the first study to compare cost-effectiveness option of adjuvant chemotherapy for colon cancer between FOLFOX4 and XELOX. The authors found that XELOX could achieve the maximum level of clinical benefit over other adjuvant treatments, while being a more affordable option in China.

Applications

This result is worthy of consideration for both doctors and patients, and provides decision makers a more comprehensive view of treatment-related cost-effectiveness in clinical practice.

Terminology

Five states were included in our Markov model and states 1 to 5 were as follows orderly: well, minor toxicity, major toxicity, quitting adjuvant chemotherapy, and death due to adjuvant chemotherapy. Transitions among the 5 states were assumed to be Markovian. Pw-w, Pw-mi, Pw-ma, Pmi-w, Pmi-mi, Pmi-ma, Pma-w, Pma-mi, Pma-ma, Pma-q, and Pma-d were applied to denote the probabilities of transition of the model in which suffix w represented the first state (well state), mi represented state of minor toxicity, ma represented state of major toxicity, q represented state of quitting the adjuvant chemotherapy, and d represented death state due to the adjuvant chemotherapy. Pw-mi represented the probability of the changing of patient in the well state in the current cycle to the minor toxicity state in the next cycle.

Peer review

In this cost-effectiveness analysis, the authors compared XELOX and FOLFOX4 as adjuvant chemotherapy for patients with colon cancer based on data obtained from MOSAIC and No. 16968 trails from a Chinese cost-effectiveness perspective. The authors demonstrated that XELOX was expected to dominate FOLFOX4 regimens; in other words, XELOX was a more cost-effective treatment for adjuvant chemotherapy for patients with colon cancer in China.

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CLINICAL TRIALS STUDY

Ultrasound hepatic/renal ratio and hepatic attenuation rate for quantifying liver fat content

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Abstract

AIM: To establish and validate a simple quantitative assessment method for nonalcoholic fatty liver disease (NAFLD) based on a combination of the ultrasound hepatic/renal ratio and hepatic attenuation rate.

METHODS: A total of 170 subjects were enrolled in this study. All subjects were examined by ultrasound and ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) on the same day. The ultrasound hepatic/renal echo-intensity ratio and ultrasound hepatic echo-intensity attenuation rate were obtained from ordinary ultrasound images using the MATLAB program.

RESULTS: Correlation analysis revealed that the ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate were significantly correlated with ^{1}H-MRS liver fat content (ultrasound hepatic/renal ratio: r = 0.952, P = 0.000; hepatic echo-intensity attenuation r = 0.850, P = 0.000). The equation for predicting liver fat content by ultrasound (quantitative ultrasound model) is: liver fat content (%) = 61.519 × ultrasound hepatic/renal ratio + 167.701 × hepatic echo-intensity attenuation rate -26.736. Spearman correlation analysis revealed that the liver fat content ratio of the quantitative ultrasound model was positively correlated with serum alanine aminotransferase, aspartate aminotransferase, and triglyceride, but negatively correlated with high density lipoprotein cholesterol. Receiver operating characteristic curve analysis revealed that the optimal point for diagnosing fatty liver was 9.15% in the quantitative ultrasound model. Furthermore, in the quantitative ultrasound model, fatty liver diagnostic sensitivity and specificity were 94.7% and 100.0%, respectively, showing that the quantitative ultrasound model was better than conventional ultrasound methods or the combined ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate. If the ^{1}H-MRS liver fat content had a value < 15%, the sensitivity and specificity of the ultrasound quantitative model would be 81.4% and 100%, which still shows that using the model is better than the other methods.

CONCLUSION: The quantitative ultrasound model is a simple, low-cost, and sensitive tool that can accurately assess hepatic fat content in clinical practice. It provides an easy and effective parameter for the early diagnosis of mild hepatic steatosis and evaluation of the efficacy of NAFLD treatment.

Key words: Non-alcoholic fatty liver disease; Ultrasound hepatic/renal ratio; Ultrasound hepatic echo-intensity attenuation rate

Core tip: The quantitative ultrasound model is a simple, low-cost, and sensitive tool that can accurately assess hepatic fat content in clinical practice. It provides an easy and effective parameter for early diagnosis of mild hepatic steatosis and evaluation of the efficacy of

non-alcoholic fatty liver disease treatment.

Zhang B, Ding F, Chen T, Xia LH, Qian J, Lv GY. Ultrasound hepatic/renal ratio and hepatic attenuation rate for quantifying liver fat content. *World J Gastroenterol* 2014; 20(47): 17985-17992 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17985.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17985

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most important causes of chronic liver disease. At present, the prevalence of NAFLD has been increasing year by year, reaching 30% in Europe and the United States and about 15% in Asia[1]. NAFLD is closely associated with the occurrence of many chronic diseases, such as diabetes and cardiovascular diseases. NAFLD was also reported to be associated with increased insulin resistance[2]. A meta-analysis showed that the prevalence of diabetes significantly increased in patients with NAFLD, and was 2-3 times higher than normal[3]. If the disease is not diagnosed early, abnormal glucose metabolism and cardiovascular disease could easily manifest in patients with NAFLD. Hence, early diagnosis of NAFLD could help predict the occurrence of diabetes and cardiovascular disease, which is important in preventing these chronic diseases from developing further.

At present, the gold standard for diagnosing NAFLD is liver biopsy. However, liver biopsy is invasive and may cause serious complications - a method not suitable for screening NAFLD. ^{1}H magnetic resonance spectroscopy (^{1}H-MRS) is a noninvasive technique that can quantitatively detect NAFLD with high sensitivity. However, using this technique is not practical for screening NAFLD, due to equipment requirements and high cost[4]. Therefore, it appears that there is an urgent need for screening early NAFLD by using simpler and more efficient methods.

Liver ultrasonic scanning is one of the common methods for diagnosing NAFLD, but this method can be easily affected by subjective factors; hence, this method cannot accurately detect liver fat content. As a result, it is difficult to diagnose NAFLD at an early stage. However, with the development of computer technology, it is now possible to quantitatively determine liver fat content by the ultrasound hepatic/renal ratio. Xia *et al*[5] found that the ultrasound hepatic/renal ratio significantly correlated with liver biopsy and ^{1}H-MRS in determining liver fat content, which implied that the ultrasound hepatic/renal ratio could partly reflect NAFLD development. In addition, the hepatic echo-intensity attenuation rate is an ultrasonic quantitative indicator; and Kwon *et al*[6] found that the hepatic echo-intensity attenuation rate showed a correlation with the degree of NAFLD in animal experiments; however, results were obtained by applying these two methods had low accuracy. Szczepaniak *et al*[7] found that the sensitivity and specificity of the ultrasound hepatic/renal ratio (62.7%) and the hepatic echo-intensity attenuation rate (68.0%) in fatty liver diagnosis were 64.7% and 70.0%, respectively. Sensitivity and specificity were still not able to achieve the requirements for clinical diagnosis of NAFLD. Physicians need a mature quantitative ultrasound model that can provide simple, accurate, and stable results in evaluating liver fat content.

Hence, in our hypothesis, we propose a combined ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate evaluation; compensating for their shortcomings and improving the accuracy of detecting NAFLD. This study aims to explore a simple, low-cost, and sensitive model that can detect liver fat content. The specificity and sensitivity of this new evaluation model were determined.

MATERIALS AND METHODS

Subjects

From December 2012 to October 2013, 129 patients with NAFLD in our hospital were enrolled according to the following inclusion criteria: (1) diagnosed with fatty liver by ultrasound; (2) no recent heavy drinking history; (3) did not take drugs that could affect liver functions and liver fat; and (4) no serious liver or kidney disease.

The 129 NAFLD patients had a mean age of 45.3 ± 7.1 years; 83 cases were diagnosed with mild fatty liver and 46 cases were diagnosed with severe fatty liver. The 41 healthy subjects diagnosed without fatty liver were enrolled in the control group.

Methods

Anthropometric measurement and biochemical detection: The height, weight, waistline, and hipline measurements were taken from the patients; body mass index (BMI) and waist/hip ratios were then calculated. Triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and liver enzyme, alanine and aspartate aminotransferase (AST, ALT) serum levels were recorded.

Fat content detection by ^{1}H-MRS: The liver fat content of all subjects were detected by ^{1}H-MRS. The right lobe of the liver was located when patients were lying in the supine position. Areas under the water peak and fat peak were recorded. Liver fat content was calculated as [liver fat content (%) = area under the fat peak × 100/(area under the fat peak + area under the water peak)]. Liver fat content ⩾ 5.56% was defined as fatty liver[7-9].

Ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate analysis: All subjects underwent color Doppler ultrasound examinations. Ultrasonic images were analyzed by MATLAB software. Average gray-scale intensities were determined in liver and renal cortex regions of interest (ROIs). The hepatic/renal ratio

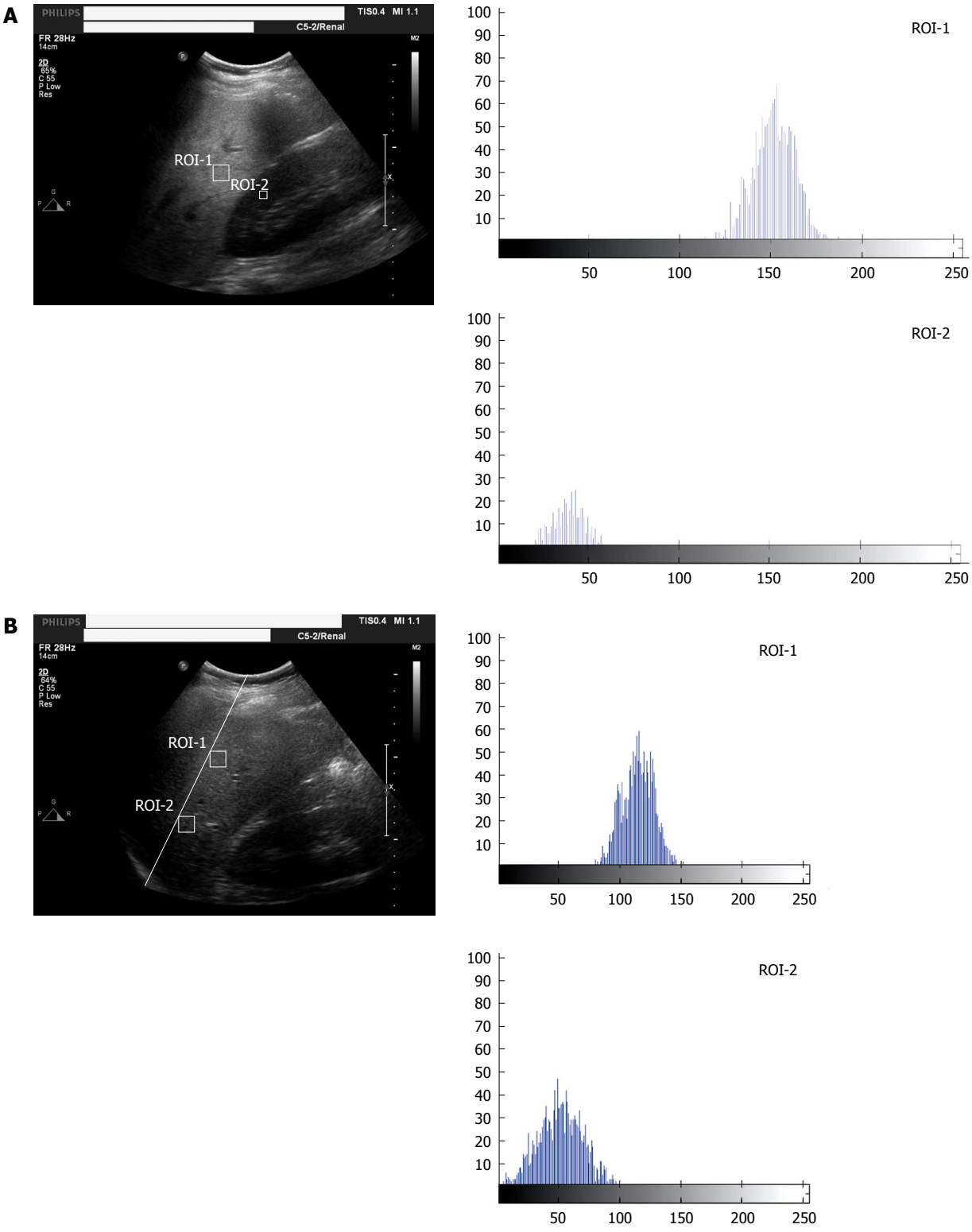

Figure 1 Ultrasonic liver image with hepatic/renal ratio and hepatic echo-intensity attenuation rate. A: Ultrasonic liver image with hepatic/renal ratio. ROI-1 and ROI-2 stands for the echo gray histograms of the liver and kidney cortex ROIs; B: Ultrasonic liver image with hepatic echo-intensity attenuation rate. ROI-1 and ROI-2 stands for the near-field and far-field echo gray histograms of the liver. ROI: Region of interest.

was calculated according to the equation: hepatic/renal ratio = mean gray-scale intensity of the liver/mean gray-scale intensity of the renal cortex (Figure 1A). Two liver ROI samples were selected at a depth of 4-6 cm from the near-field of the same beam. The distance between the two ROIs were measured (Figure 1B). The hepatic echo-intensity attenuation rate was calculated according to the equation: hepatic echo-intensity attenuation rate = (lnAn-lnAf)/(Δd × f); where An and Af represent the mean echo intensity of the near-field and far-field ROIs,

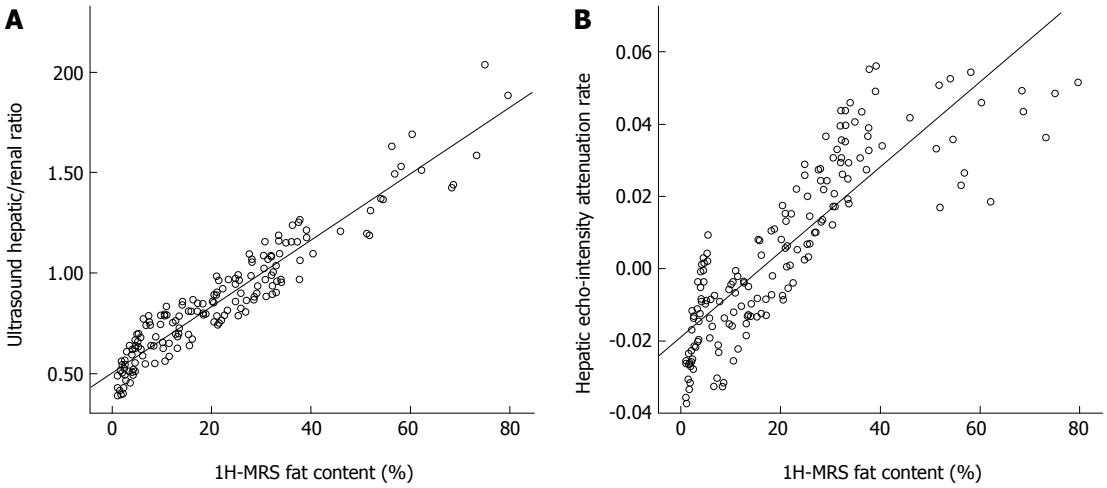

Figure 2 Linear correlation analysis between ^{1}H-magnetic resonance spectroscopy liver fat content and ultrasound hepatic/renal ratio (A), and hepatic echo-intensity attenuation rate (B). The illustration shows that the ^{1}H-MRS liver fat content that was determined significantly correlated with (A) the ultrasound hepatic/renal ratio (r = 0.952, P = 0.000) and (B) the hepatic echo-intensity attenuation rate (r = 0.850, P = 0.000). MRS: Magnetic resonance spectroscopy.

Table 1 General data comparison of the two groups

Classification	Non-fatty liver group (n = 41)	Fatty liver group (n = 129)	P
Age (yr)	54.32 ± 6.24	53.72 ± 5.81	0.182
Gender (male/female)	20/21	67/62	0.613
BMI (kg/m^2)	24.11± 0.73	27.38 ± 0.64	0.005
Waist/hip ratio	0.86 ± 0.02	0.93 ± 0.01	0.001
ALT (IU/L)	18 (11-22)	35 (18-41)	0.001
AST (IU/L)	18 (14-25)	24 (18-39)	0.001
TC (mmol/L)	1.03 (0.75-1.22)	1.72 (1.35-2.43)	0.000
TG (mmol/L)	4.87 ± 0.27	5.02 ± 0.18	0.532
HDL-C (mmol/L)	1.42 ± 0.05	1.18 ± 0.04	0.003
LDL-C (mmol/L)	3.01 ± 0.23	2.98 ± 0.15	0.672
Ultrasound hepatic/renal ratio	0.55 ± 0.01	0.80 ± 0.04	0.000
Hepatic echo-intensity attenuation rate (cm^{-1}·MHz^{-1})	-0.0193 ± 0.0031	0.0027 ± 0.0016	0.000

BMI: Body mass index; TG: Triglyceride; TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.

respectively; Δd is the line distance between the two ROIs and f is the ultrasonic transducer frequency.

Statistical analysis

Data was analyzed using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, United States). Numerical variable data with normal distribution were recorded as (mean ± SD). Spearman correlation analysis was performed between the quantitative ultrasound parameters and ^{1}H-MRS liver fat content. The optimum qualitative diagnosis point of the NAFLD and quantitative fat content estimation by ultrasound were determined by receiver operating characteristic (ROC) curve analysis. The optimum ultrasonic quantity point was confirmed by the Youden index [maximum value of (sensitivity + specificity-1)]. Logistic regression analysis was used for the quantitative ultrasound parameters to determine the equation for predicting liver fat content. $P < 0.05$ indicates that the differences were statistically significant.

RESULTS

Analysis of the subject's general data

Based on the diagnostic standard (liver fat content ⩾ 5.56% defined as fatty liver) determined by ^{1}H-MRS, the subjects were divided into two groups, the fatty liver group and the non-fatty liver group. The general data of the two groups was compared. BMI, waist/hip ratio, and serum ALT, AST, and TC levels of the fatty liver group were significantly higher than the non-fatty liver group, while serum HDL-C levels were significantly lower. The differences were statistically significant ($P < 0.05$). The age, gender, TG, and LDL-C differences between the two groups were not statistically significant ($P > 0.05$). The ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate of the fatty liver group were significantly higher than the non-fatty liver group; and the differences were statistically significant ($P < 0.05$) (Table 1).

Correlation between quantitative ultrasound parameters and liver fat content determined by ^{1}H-MRS

Correlation analysis revealed that the ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate were significantly correlated with ^{1}H-MRS liver fat content (ultrasound hepatic/renal ratio: r = 0.952, P = 0.000; hepatic echo-intensity attenuation r = 0.850, P = 0.000) (Figure 2).

Defining the quantitative ultrasound model for liver fat content estimation

^{1}H-MRS liver fat content was set as a dependent variable. Multiple linear regression analysis was utilized to screen the main quantitative parameters for liver fat content estimation. The ultrasound hepatic/renal ratio was the stron-

Table 2 Quantitative ultrasound index estimation model for liver fat content

	Model	Regression coefficients ± SD				*P* value	Corrective R^2	RMSE
		Ultrasound hepatic/renal ratio	Hepatic echo-intensity attenuation rate	Constant				
Liver fat content	1	72.012 ± 3.445	-	-34.704 ± 2.302	-34.704 ± 2.302	0.000	79.0%	6.12
	2	61.519 ± 4.311	167.701 ± 42.115	-26.736 ± 3.012	-26.736 ± 3.012	0.000	80.1%	5.33

Model 1: Ultrasound hepatic/renal ratio model to estimate ^{1}H-MRS liver fat content. Model 2: Hepatic echo-intensity attenuation rate model to estimate ^{1}H-MRS liver fat content. RMSE: Root mean square error; ^{1}H-MRS: ^{1}H-magnetic resonance spectroscopy.

Table 3 Correlation analysis between metabolic indices and liver fat content determined by the quantitative ultrasound model

	AST	ALT	TC	TG	HDL-C	LDL-C
r	0.301	0.411	0.015	0.512	-0.331	-0.079
P	0.001	0.000	0.721	0.000	0.001	0.332

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TG: Triglyceride; TC: Total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.

gest predictor of liver fat content (corrective R^2 = 79.0%, P = 0.000). When combined with the hepatic echo-intensity attenuation rate, a higher estimation accuracy can be achieved (corrective R^2 = 80.1%, P = 0.000). The equation for liver fat content prediction by ultrasound (quantitative ultrasound model) was: liver fat content (%) = 61.519 × ultrasound hepatic/renal ratio + 167.701 × hepatic echo-intensity attenuation rate -26.736 (Table 2).

Correlation analysis between the metabolic indices and the liver fat content quantitative ultrasound model

Spearman correlation analysis revealed that the liver fat content quantitative ultrasound model was positively correlated with serum ALT, AST, and TG, but negatively correlated with HDL-C; however, it was not correlated with serum LDL-C and TC (Table 3).

Fatty liver diagnosis by the quantitative ultrasound model and conventional ultrasound

ROC analysis revealed that the optimum point of fatty liver diagnosis was 9.15%, using the quantitative ultrasound model. When ^{1}H-MRS was set as the gold standard for diagnosing fatty liver by the quantitative ultrasonic model, the sensitivity and specificity for fatty liver diagnosis were 94.7% and 100.0%, respectively. These results were better than using the conventional ultrasound method, or single use of the ultrasound hepatic/renal ratio and the hepatic echo-intensity attenuation rate. A subgroup analysis was also performed, where ^{1}H-MRS liver fat content was < 15%; the results for the sensitivity and specificity of the quantitative ultrasound model were 81.4% and 100%, respectively. These results are still better than applying other methods (Table 4).

DISCUSSION

In this study, the ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate significantly correlated with ^{1}H-MRS liver fat content. The sensitivity and specificity of fatty liver diagnosis by the novel quantitative ultrasound model (hepatic/renal ratio and hepatic echo-intensity attenuation rate combination) were significantly higher than by conventional ultrasound; which implies that the quantitative ultrasound model can conveniently and accurately determine liver fat content. We hope that this model could provide a convenient and effective method for diagnosing NAFLD and mild fatty liver degeneration.

Correlation between ultrasound hepatic/renal ratio, hepatic echo-intensity attenuation rate, and ^{1}H-MRS liver fat content

Currently, ^{1}H-MRS is considered the gold standard for determining liver fat content[10,11]. In this study, the hepatic/renal ratio and hepatic echo-intensity attenuation rate were highly correlated with ^{1}H-MRS liver fat content; suggesting that these methods could also accurately assess liver fatty content as well. Compared with ^{1}H-MRS, these methods are more convenient, economic, and easy to operate[3,12,13], which can be used as new tools for clinical NAFLD diagnosis.

Efficacy comparison of the quantitative ultrasound model and conventional ultrasound in fatty liver diagnosis

This study shows that the sensitivity and specificity of fatty liver diagnosis, by the ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate, were significantly better than the conventional ultrasound method. Furthermore, combining the two methods and the quantitative ultrasound model resulted in better sensitivity and specificity, which implies that the quantitative ultrasound model could significantly improve the sensitivity and specificity of fatty liver diagnosis. A possible reason for this observation is that the traditional standard for ultrasound fatty liver diagnosis is relatively subjective and

Table 4 Comparison of fatty liver diagnosis by the ultrasound quantitative model, the ultrasound hepatic/renal ratio, the hepatic echo-intensity attenuation rate, and conventional ultrasound

Group	^{1}H-MRS diagnosis of fatty liver			
	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
All subjects				
Quantitative ultrasound model	94.7	100.0	100.0	82.6
Conventional ultrasound	83.7	70.7	90.0	58.0
Ultrasound hepatic/renal ratio	88.3	84.5	94.2	72.4
Hepatic echo-intensity attenuation rate	86.7	78.7	92.0	67.0
Subjects with fatty content < 15% (^{1}H-MRS)				
Quantitative ultrasound model	81.4	100.0	100.0	84.5
Conventional ultrasound	47.6	70.3	73.1	62.7
Ultrasound hepatic/renal ratio	67.5	81.7	87.6	70.3
Hepatic echo-intensity attenuation rate	55.6	74.3	82.1	68.7

^{1}H-MRS: ^{1}H-magnetic resonance spectroscopy.

can easily result in misdiagnosis[14-16]. However, a computer-assisted quantitative ultrasound model makes the fatty liver objective and quantitative, which cannot easily be influenced by subjective factors and avoids subjective deviation[5,17,18]. Meanwhile, this method can accurately provide quantitative liver fat content information; overcoming the limitations of a qualitative-only diagnosis performed by conventional ultrasound.

Efficiency of the quantitative ultrasound model in mild fatty liver diagnosis

The result shows that the sensitivity and specificity of mild fatty liver diagnosis by quantitative ultrasound model is significantly higher than by ultrasound hepatic/renal ratio, hepatic echo-intensity attenuation rate, or conventional ultrasound. The result also indicates that even with a slightly echo-enhanced ultrasound image of the liver, fatty liver still could not be distinguished by the naked eye; but fatty liver could be identified early by using the indices of the quantitative ultrasound model. Hence, patients with early fatty liver can be diagnosed by using the quantitative ultrasound model[19,20]. Moreover, the quantitative ultrasound model is more convenient and easier to operate than the ^{1}H-MRS, especially for early fatty liver screening in a large population.

Correlation analysis between metabolic indices and fat content by the quantitative ultrasound model

The study showed that liver fat content determined by quantitative ultrasound model was positively correlated with serum ALT, AST, and TG, but negatively correlated with HDL-C; suggesting a correlation with liver function and blood lipids. The result was similar to the previous studies on determining liver fat content by ^{1}H-MRS[21-23]. Most patients diagnosed with fatty liver have liver function damage and dyslipidemia. The quantitative ultrasound model can detect this correlation, and we hope that this method could be a useful tool for detecting fatty liver in clinical practice.

Limitation

There are still some deficiencies in this study. At present, the strictest standard for diagnosing fatty liver is liver biopsy. In our study, ^{1}H-MRS detection was set as the golden standard for diagnosing NAFLD and not liver biopsy, because of the high risk of complications. We believe that ^{1}H-MRS is a relatively accurate and reliable method for diagnosing fatty liver, because this method was used as the diagnostic criteria for several large-scale studies. Many studies have also shown that ^{1}H-MRS was consistent with liver biopsy, in terms of diagnosing fatty liver[24-28].

In conclusion, the study successfully established the quantitative ultrasound model for liver fat content; by combining the ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate, liver fat content can be accurately determined. The study also provides a simple and effective method for the early diagnosis of mild NAFLD degeneration.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) is one of the most important causes of chronic liver disease. This disease is closely associated with the occurrence of many chronic diseases, such as diabetes and cardiovascular diseases. A meta-analysis showed that abnormal glucose metabolism and cardiovascular disease could easily manifest in patients with NAFLD, if the disease could not be diagnosed in a timely manner. Hence, early diagnosis of NAFLD could predict the occurrence of diabetes and cardiovascular disease: an important factor in preventing the development of these chronic diseases.

Research frontiers

The gold standard for NAFLD diagnosis is liver biopsy. However, this method is invasive and may cause serious complications. Furthermore, this method is not suitable for screening NAFLD. We established that liver fat content could be quantitatively detected by the ultrasound hepatic/renal ratio. Moreover, the hepatic echo-intensity attenuation rate is a quantitative ultrasound indicator. However, its sensitivity and specificity did not meet the requirement for clinically diagnosing NAFLD. Therefore, physicians need a mature quantitative ultrasound model that can provide a simple, accurate, and stable method for evaluating liver fat content.

Innovations and breakthroughs

This study successfully established the quantitative ultrasound model for determining liver fat content by combining the ultrasound hepatic/renal ratio and hepatic echo-intensity attenuation rate; which can accurately reflect liver fat content. The study also provides a simple and effective method for diagnosing early mild NAFLD degeneration.

Applications

The quantitative ultrasound model can conveniently and accurately determine

liver fat content. We hope it can provide a convenient and effective method for diagnosing NAFLD and mild fatty liver degeneration.

Peer review

This is a very interesting manuscript about ultrasound hepatic/renal ratio and hepatic attenuation rate. In this paper, the authors establish and validate a simple method for quantitative assessment of NAFLD based on the combination of ultrasound hepatic/renal ratio and hepatic attenuation rate.

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PROSPECTIVE STUDY

Poor agreement between endoscopists and gastrointestinal pathologists for the interpretation of probe-based confocal laser endomicroscopy findings

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Abstract

AIM: To compare the interpretation of probe-based confocal laser endomicroscopy (pCLE) findings between endoscopists and gastrointestinal (GI)-pathologists.

METHODS: All pCLE procedures were undertaken and the endoscopist rendered assessment. The same pCLE videos were then viewed offline by an expert GI pathologist. Histopathology was considered the gold standard for definitive diagnosis. The sensitivity, specificity and accuracy for diagnosis of dysplastic/ neoplastic GI lesions and interobserver agreement between endoscopists and experienced gastrointestinal pathologist for pCLE findings were analyzed.

RESULTS: Of the 66 included patients, 40 (60.6%) had lesions in the esophagus, 7 (10.6%) in the stomach, 15 (22.7%) in the biliary tract, 3 (4.5%) in the ampulla and 1 (1.5%) in the colon. The overall sensitivity, specificity and accuracy for diagnosing dysplastic/ neoplastic lesions using pCLE were higher for endoscopists than pathologist at 87.0% *vs* 69.6%, 80.0% *vs* 40.0% and 84.8% *vs* 60.6% (P = 0.0003), respectively. Area under the ROC curve (AUC) was greater for endoscopists than the pathologist (0.83 *vs* 0.55, P = 0.0001). Overall agreement between endoscopists and pathologist was moderate for all GI lesions (K = 0.43; 95%CI: 0.26-0.61), luminal lesions (K = 0.40; 95%CI: 0.20-0.60) and those of dysplastic/neoplastic pathology (K = 0.55; 95%CI: 0.37-0.72), the agreement was poor for benign (K = 0.13; 95%CI: -0.097-0.36) and pancreaticobiliary lesions (K = 0.19; 95%CI: -0.26-0.63).

CONCLUSION: There is a wide discrepancy in the interpretation of pCLE findings between endoscopists and pathologist, particularly for benign and malignant pancreaticobiliary lesions. Further studies are needed to identify the cause of this poor agreement.

Key words: Confocal endomicroscopy; Gastointestinal; Interobserver variation

Core tip: Probe-based confocal endomicroscopy (pCLE) has emerged as a valuable tool in the diagnosis and management of gastrointestinal disorders. It has helped the endoscopist to make real time decisions and targeted biopsies. Histopathology still remains the gold standard. We compared the interpretation of pCLE findings between an endoscopist and a dedicated gastrointestinal pathologist and found there was a discrepency in the intepretation of the same findings between them. This is interesting as the endoscopist

has a different approach of intepreting real time endomicroscopy compared to that of a pathologist.

Peter S, Council L, Bang JY, Neumann H, Mönkemüller K, Varadarajulu S, Wilcox CM. Poor agreement between endoscopists and gastrointestinal pathologists for the interpretation of probe-based confocal laser endomicroscopy findings. *World J Gastroenterol* 2014; 20(47): 17993-18000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/17993.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.17993

INTRODUCTION

Endoscopic tissue sampling with histopathology is considered the gold standard for diagnosis and management of most gastrointestinal (GI) disorders. The differentiation between benign and malignant lesions is vital to further management. Even though random biopsies are considered the norm, they are also involved in flaws such as sampling errors along with an incremental cost that may be incurred[1,2]. Confocal laser endoscopy (CLE) is a new endoscopic technology developed to obtain high resolution images of the gastrointestinal mucosa allowing *in vivo* and real time endomicroscopic analysis of the targeted tissue[3-5]. It enables differentiation between malignant and benign lesions crucial for clinical decision making. Based on defined criteria, the interpreter is able to make distinguishing decisions on the nature of the lesion for subsequent therapy[6].

The principle of CLE is based on tissue reflectance or tissue fluorescence after application of fluorescence agents (*e.g.*, fluorescein sodium) generating images that demonstrate cellular architecture and microvasculature that is comparable with traditional histology. Several studies have shown the usefulness of this technology in determining pathology in a wide range of GI tissue sites such as Barrett's esophagus (BE) (Figure 1), duodenum, colonic mucosa and pancreatic biliary lesions[7-16]. From these studies it has been shown that CLE can be performed and interpreted accurately after adequate training. However, it is still not clear what the learning curve for adequate diagnosis and interpretation using this new technology will be in predicting better outcomes. Understanding this will have long term effects on operating costs while enhancing the benefit to the patient.

In this context, we postulated that an endoscopist had the capability of real time imaging while a pathologist would have the inherent advantage of histopathological cellular differentiation. We therefore aimed to evaluate the differences in interpretation of probe-based CLE (pCLE) findings between them.

MATERIALS AND METHODS

Patient recruitment

Consecutive patients undergoing endoscopy with pCLE at a tertiary medical center were identified. Eligibility for this study consisted of an indication for endoscopy with pCLE such as Barrett esophagus, undetermined gastric or colonic polyps, ampullary neoplasms or bile duct stricture. Exclusion criteria were the following: age < 18 years, inability to give written informed consent, coagulopathy, renal failure or known allergy to fluorescein sodium. The study was approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB).

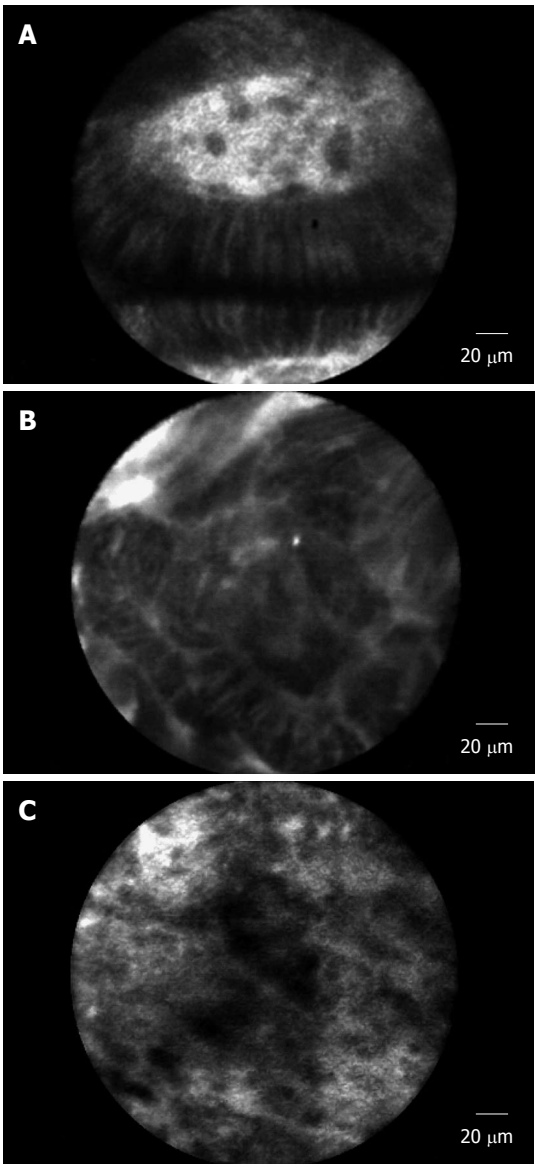

Figure 1 Probe-based confocal laser endomicroscopy images of Barrett's esophagus. A: Barrett's esophagus with intestinal metaplasia; B: Barrett's esophagus with dysplasia; C: Barrett's esophagus with neoplasia or carcinoma.

Study design

Patients underwent endoscopy (GIF; Olympus America, Center Valley, PA, United States) and were followed by pCLE (Cellvizio; Mauna Kea Technologies, Paris, France). pCLE was performed using 3 different probes appropriate for the area of pathology studied: (1) GastroFlex UHD; (2) CholangioFlex UHD; and (3) ColoFlex UHD. The probe

(diameter 2.5 mm) was inserted through the accessory channel and gently approached to the mucosa as previously descripted[5]. The depth of imaging was 40-70 mm for CholangioFlex probes, and 55-65 mm for GastroFlex and ColoFlex probes. The maximal field of view is 325 mm for Cholangio-Flex probes, 600 mm for GastroFlex and ColoFlex probes, and 240 mm for GastroFlex UHD and ColoFlex UHD. The lateral resolution is 3.5 mm for CholangioFlex and 1 mm for GastroFlex UHD and ColoFlex UHD. The images were scanned with a rate of 12 frames per second, hence demonstrating a real-time video on a second screen that is positioned next to the endoscopy monitor. For tissue contrast, 5 mL intravenous fluorescein (10%; Alcon Pharma, Novartis, E Hanover, NJ, United States) was used, which has been shown to be safe in ophthalmology and previous CLE studies[17].

Real time pCLE interpretation was rendered for all lesions after endoscopic evaluation of the area and lesion and images were stored as video sequences as well as images. pCLE image interpretation was performed according to updated Miami criteria for pCLE[6]. The results were recorded in Excel worksheets. Subsequent biopsies were taken from all studied areas; these were collected after detection of the lesion, after interpretation of the image *via* pCLE. Histologic samples were processed by using standard procedures and evaluated by an expert pathologist specialized in gastroenterology. Biopsies were classified at histology according to the type of epithelium (inflammatory or hyperplastic polyp, tubular, tubulovillous, or villous adenoma) and degree of dysplasia (none, low-grade, high-grade, or cancer) according to the updated Vienna criteria for the diagnosis of GI neoplasia, omitting the category moderate dysplasia[18]. The histologic slides were separately reviewed by a dedicated gastrointestinal pathologist. Histology was considered the gold standard for diagnosis.

Video and image evaluation

Learning phase: In order to standardize image interpretation the gastroenterologists (and the GI pathologist underwent special training with formal certification in defining criteria of lesions using pCLE according to updated Miami classification[6]. Therefore, a total of 20 videos and image sets were used to train the participants using a standardized training set from Cellvizio, Mauna Kea Technologies. Some of the topics of the videos included lesions from the esophagus *e.g.*, Barrett's (normal or neoplastic), colonic lesions (hyperplastic *vs* neoplastic) or pancreaticobiliary lesions. The training included didactic teaching involving approximately 6 h and culminating in an exam format. All endoscopists were naïve to this tool similarly the pathologist had no previous experience.

Practice phase: The three endoscopists performed a total of 70 cases of pCLE during the study period.

Study phase: Image selection and image evaluation was done and a total of 70 video clips with images of consecutive patients were selected. First, the endoscopist rated the histological diagnosis based on pCLE findings and results were entered in Excel worksheets. Accordingly, the pCLE videos were viewed offline by the expert GI pathologist who was blinded to the image interpretation of the endoscopist. The videos were defined as "benign" or "dysplasia/neoplasia" if they contained dysplasia or cancer. From the selected consecutive pCLE videos, the overall impression of the interpreters was evaluated for any of the above changes (Figure 2). If images were rendered not clear or difficult to interpret, this was also included in the analysis.

Statistical analysis

Data from the study were entered into a Microsoft Excel (Microsoft Corporation, Washington, United States) spreadsheet. Information from the GI physicians as well as the GI pathologist was entered onto a case report form and further entered into the Excel database. The accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for prediction of benign/normal *vs* dysplasia/neoplasia was calculated between them. Interobserver variability was calculated using the *K* statistic and results based on this were defined as: poor < 0.2, fair 0.21-0.4, moderate 0.41-0.6, substantial 0.61- 0.8 and excellent 0.81-1[19]. Statistical analysis was performed using SAS 9.2 (SAS Institute, Cary, NC, United States).

RESULTS

A total of 70 consecutive patients undergoing pCLE imaging were included in the study, 4, patients were excluded because of incomplete information or poor image files and quality (Figure 3). Sixty-six patients were finally included, male 66.7%, female 33.3%, mean age were 60.3 ± 13.6 years. Majority of patients underwent pCLE for determination of indeterminate lesions 34 (51.5%) and surveillance 18 (27.3%) (Table 1). Of 66 patients, 40 (60.6%) had lesions in the esophagus, 7 (10.6%) in the stomach, 15 (22.7%) in the biliary tract, 3 (4.5%) in the ampulla and 1 (1.5%) in the colon (Table 1). The pathologies included Barrett's esophagus, colonic and gastric polyps with indeterminate pre-pCLE diagnosis, indeterminate biliary strictures, and ampullary lesions.

The overall sensitivity, specificity and accuracy for diagnosing dysplastic/neoplastic lesions using pCLE were higher for endoscopists than pathologist at 87.0% *vs* 69.6%, 80.0% *vs* 40.0% and 84.8% *vs* 60.6% ($P = 0.0003$), respectively. For luminal lesions (esophagus, stomach and colon) they were 82.4% *vs* 64.7%, 92.9% *vs* 42.9% and 85.4% *vs* 58.3%. For ampullary and pancreaticobiliary lesions the results were 100% *vs* 83.3%, 50% *vs* 33.3% and 100% *vs* 50% (Table 2). Also, the area under the ROC curve (AUC) was greater for endoscopists than the pathologist (0.83 *vs* 0.55, $P = 0.0001$) (Figure 4).

While the overall agreement between endoscopists and pathologist was moderate for all GI lesions ($K = 0.43$;

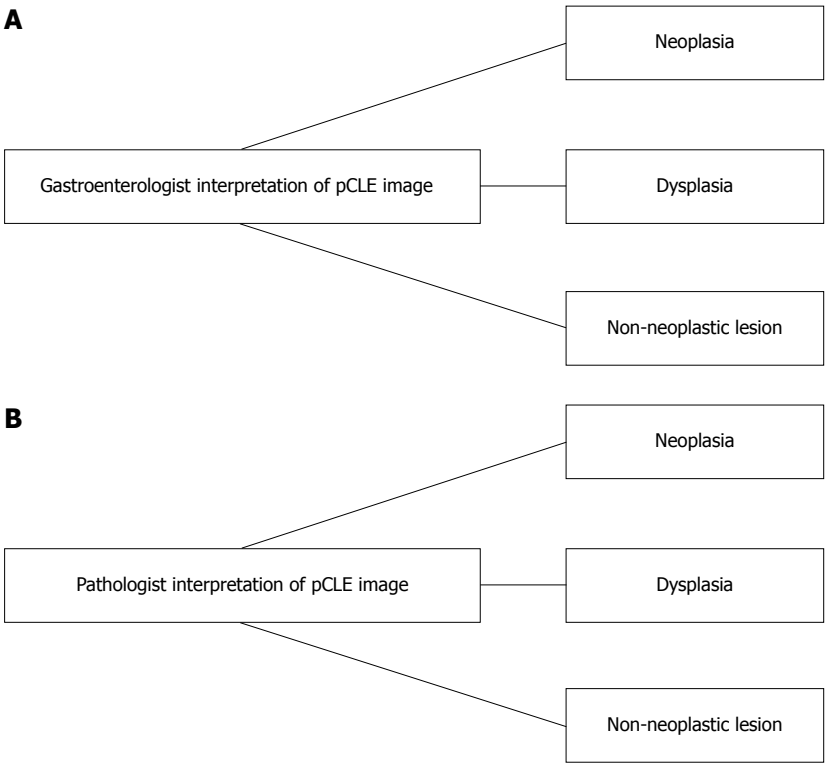

Figure 2 Flow chart for interpretation of results. A: Anatomical site; B: Interpretation of Cellvizio image. pCLE: Probe-based confocal laser endomicroscopy.

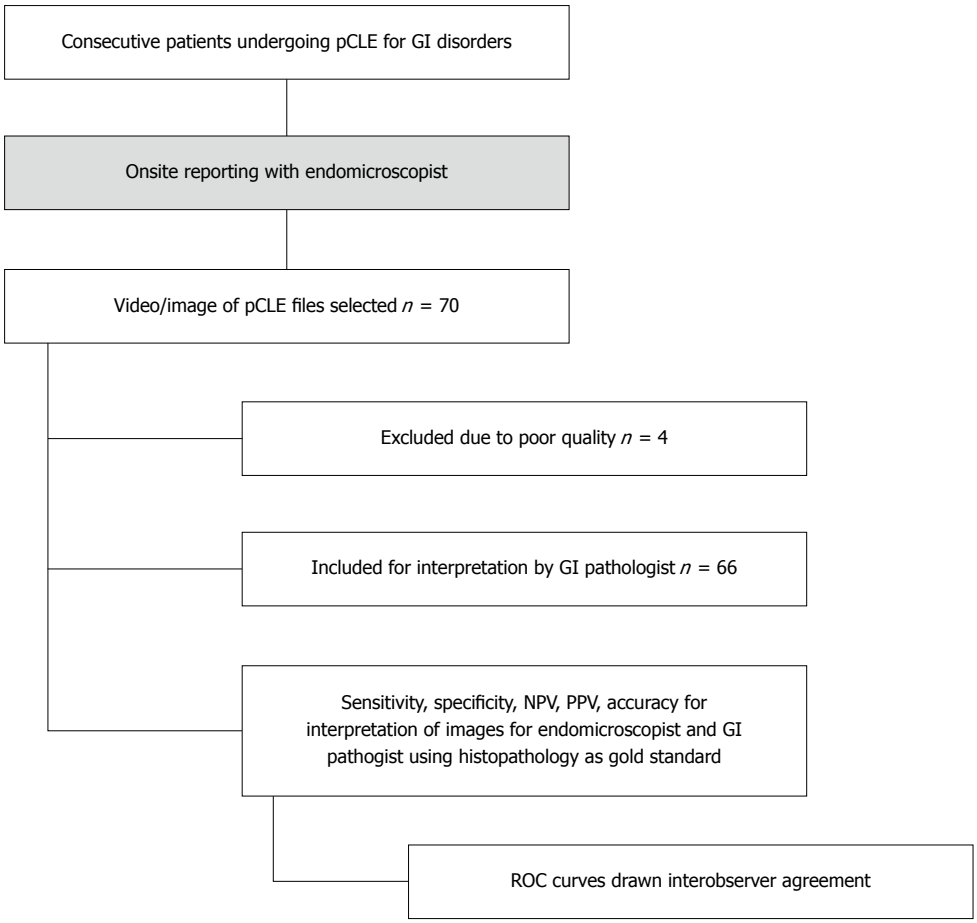

Figure 3 Flowchart of patient recruitment. pCLE: Probe-based confocal laser endomicroscopy; PPV: Positive predictive value; NPV: Negative predictive value.

Table 1 Summary of patient and lesions characteristics *n* (%)

Characteristics			
Age (yr)	Mean ± SD		60.3 ± 13.6
	Median		61.5
	IQR		51-71
	Range		17-86
Gender	Female		22 (33.3)
	Male		44 (66.7)
Race	Black		7 (10.6)
	White		54 (81.8)
	Other		5 (7.6)
Lesion type	Esophagus	All	40 (60.6)
		Normal/Benign	12 (30)
		LGD	13 (32.5)
		HGD	8 (20)
		Neoplastic	7 (17.5)
	Gastric	All	7 (10.6)
		Normal/Benign	2 (28.6)
		LGD	1 (14.3)
		HGD	2 (28.6)
		Neoplastic	2 (28.6)
	Colonic	All	1 (1.5)
		Normal/Benign	0
		LGD	0
		HGD	1 (100)
		Neoplastic	0
	Ampulla	All	3 (4.5)
		Normal/Benign	2 (66.7)
		LGD	0
		HGD	1 (33.3)
		Neoplastic	0
	Pancreaticobiliary	All	15 (22.7)
		Normal/Benign	4 (26.7)
		LGD	0
		HGD	1 (6.7)
		Neoplastic	10 (66.7)
Indication for pCLE	Indeterminate lesion		34 (51.5)
	Surveillance		18 (27.3)
	Targeted biopsy ± therapy		13 (19.7)
	Other		1 (1.5)

pCLE: Probe-based confocal laser endomicroscopy.

95%CI: 0.26-0.61), luminal lesions (K = 0.40; 95%CI: 0.20-0.60) and those of dysplastic/neoplastic pathology (K = 0.55; 95%CI: 0.37-0.72), the agreement was poor for benign (K = 0.13; 95%CI: -0.097-0.36) and pancreatico-biliary lesions (K = 0.19; 95%CI: -0.26-0.63) (Table 3).

DISCUSSION

In this study we found that there was inconsistent agreement between endoscopists and an experienced GI pathologist for the diagnosis of GI lesions using pCLE. While the agreement for the diagnosis of dysplastic or malignant lesions was moderate the concordance for benign lesions was suboptimal.

There are several potential reasons that may explain these findings. It could be argued that endoscopists have more practice reading endomicroscopy. This assumption is unlikely, as pCLE is a relatively new technology and its interpretation requires knowledge of microstructural (*i.e.*,

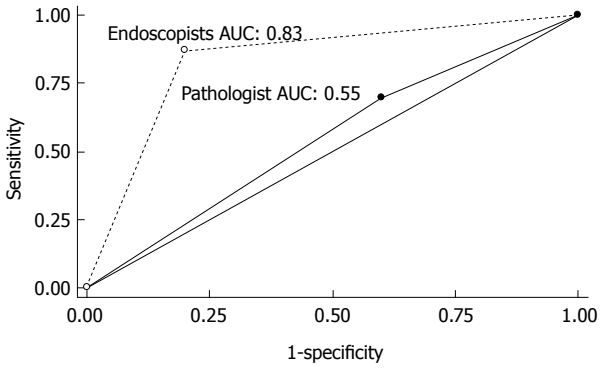

Figure 4 Receiver operating characteristic curves for endoscopists and pathologist for diagnosis of dysplastic/neoplastic lesions using probe-based confocal laser endomicroscopy.

pathological) changes. We hypothesized that the pathologist will have the "natural" advantage of understanding the cell structure given their expertise in cyto-pathological interpretation. This is one of the aspects that make our study important, as we have shown that interpretation of images is not solely based on ultra-structural knowledge. In addition, the interpretation is not only based on still photos, but relies on video sequences as well. This could be a challenge for a pathologist, who loses the ability to "control" the slide specimen. Therefore, our study has clinical implications as it emphasizes the necessity to implement and focus on training in interpretation of pCLE images and videos.

A crucial aspect of standard pathology is the ability to archive tissue and slides for future re-analysis and processing. With pCLE there is also a possibility of storing the "specimen" (*i.e.*, imaged sequences) for future analysis. This feature is of paramount importance as often a clear-cut diagnosis will not be established during live endoscopy and review of the data will clarify or allow the clinician to reach a diagnosis. It is also possible that the endoscopist has an inherent advantage of achieving a diagnosis while performing the endoscopy. In clinical practice there is usually a flood of clinical, laboratory and radiological data that aids the physician in reaching a diagnosis. In addition, during endoscopy, there are additional features observed that can improve the diagnostic yield. It could be argued that endoscopists had an advantage as they were performing doing regular endomicroscopy and thus had more skills to interpret the images when confronted with them during the study phase. In contrast, the GI pathologist could be considered relatively a "non-expert" given the experience with documented didactic training on confocal endomicroscopy and had no onsite endoscopic real time expertise. Of note, we did not try to replace their individual potential roles however aimed to see how best these results were in relevance to enhance the overall reading performance of confocal endomicroscopy and in effect reduce the overall biopsy burden by specific targeting of relevant areas. We theorized that such an analysis would help in tailoring and understanding their specific roles

Table 2 Comparison of diagnostic accuracy between endoscopists and pathologist

	Lesion site	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Accuracy (95%CI)
Endoscopist	All lesion types	87.0 (77.2-96.7)	80.0 (62.4-97.5)	90.9 (82.4-99.4)	72.7 (54.1-91.3)	84.8 (76.2-93.5)
	Luminal[1]	82.4 (69.5-95.2)	92.9 (66.1-99.8)	96.6 (82.2-99.9)	68.4 (47.5-89.3)	85.4 (75.4-95.4)
	Ampulla/PB	100 (73.5-100)	50.0 (11.8-88.2)	80.0 (51.9-95.7)	100 (29.2-100)	83.3 (58.6-96.4)
Pathologist	All lesion types	69.6 (56.3-82.9)	40.0 (18.5-61.5)	72.7 (59.6-85.9)	36.4 (16.3-56.5)	60.6 (48.8-72.4)
	Luminal[1]	64.7 (48.6-80.8)	42.9 (16.9-68.8)	73.3 (57.5-89.2)	33.3 (11.6-55.1)	58.3 (44.4-72.2)
	Ampulla/PB	83.3 (51.6-97.9)	33.3 (4.3-77.7)	71.4 (41.9-91.6)	50.0 (6.8-93.2)	66.7 (41.0-86.7)
P value for overall rates:		-	-	-	-	< 0.001[2]
P value for luminal lesions:		-	-	-	-	< 0.001[2]
P value for PB lesions:		-	-	-	-	0. 375[2]

[1]Luminal lesions include lesions located in the esophagus, stomach and colon. [2]*P* values were calculated using the McNemar's test to compare the diagnostic accuracy between endoscopist and pathologist. *P* values cannot be calculated for sensitivity, specificity, NPV or PPV. PPV: Positive predictive value; NPV: Negative predictive value; PB: Pancreaticobiliary.

Table 3 Inter-observer variation between endoscopists and pathologist

		Agreement	kappa	*P* value
Overall (all lesion types)		73.7%	0.43	< 0.001
Lesion site:	Luminal	75.0%	0.40	< 0.001
	Ampulla/PB	70.4%	0.19	0.191
Degree of abnormality	Benign	60.0%	0.13	0.102
	Dysplasia	84.0%	0.58	< 0.001
	Neoplasia	73.7%	0.07	0.114
	Dysplasia/ neoplasia combined	79.7%	0.55	< 0.001

within the multidisciplinary approach to further treatment.

Also, in our study we also wanted to reflect real practice and therefore selected to have a wide variety of pathologies to review. Thus, our study was unique such that images designated and studied were well dispersed among a varied spectrum of disorders including benign to malignant gastrointestinal lesions such as Barrett's esophagus, gastric polyps, colonic polyp, ampulla tumors and intrabiliary indeterminate strictures.

From the results, the endomicroscopists scored significantly better in overall interpretation of lesions than the pathologist with higher accuracy, sensitivity and specificity. It seems to suggest that despite the advantage of having cellular interpretation the pathologist wasn't good for interpreting confocal images. This could be related to the tangential view as to how the cells are oriented while imaging with the probe base laser device as compared to routine cross sectional microscopy for routine histopathology. Secondly, the specific cell HE staining for cellular morphology is not possible during endomicroscopy. Thirdly, there is a disadvantage of not able to visualize these in real - time with close details of the lesions within sight which would have resulted in higher accuracy reported in the endomicroscopy group.

In a previous study Dunbar *et al*[20] evaluated whether the combined use of CLE and pathology improved the diagnostic yield of Barrett esophagus-associated neoplasia as compared to standard endoscopy with four-quadrant biopsy protocol. Although the authors found that targeted biopsy using CLE reduces the amount of biopsies needed to make a diagnosis and significantly improved the diagnostic yield for endoscopically in and apparent BE neoplasia, the overall k value for all participants was also moderate at 0.56 (95%CI: 0.50-0.62). Their study aimed primarily at evaluating the utility of CLE for predicting mucosal histopathology. They could not assess accuracy because mucosal biopsy was not routinely performed during the CLE procedure and *in vivo* CLE imaging did not show HGD or cancer.

In another study by Gaddam *et al*[21] predefined pCLE criteria were tested to evaluate the difference in interpretation of Barrett's esophagus between experts and non-experts consisting of all gastroenterologists. They found that the accuracy in diagnosing dysplasia was 81.5 % (95%CI: 77.5%-81%), with no difference between experts *vs* non-experts k = 0.61 (0.53-0.69), suggesting that both groups could interpret these images after a short learning curve. Based on the results from our study we believe that the skills to learn pCLE may be acquired more slowly and depend on real-life endoscopy. The endoscopist has the benefit to control the scope, the angles of visualization and the knowledge of what they saw. Also the pathologist did not view the endoscopic images enabling the endoscopist to have more elements for the diagnosis as mentioned. These findings are of additional importance as it would imply that regular experience of live cases or continuous exposure to pCLE would be important for a pathologist to acquire more skills in the interpretation of images. The availability of images and/or clips could enhance the pathologist's ability to make

a specific diagnosis. This demonstrates the importance of joint collaboration and work of endoscopists and GI pathologists. It is well known that close collaboration during EUS-guided fine needle aspiration (FNA) and on-site interpretation of cytological specimens improves the diagnostic accuracy. This concept may need to be applied to the training phase of CLE, thus inviting the pathologist to participate during live endoscopy and acquire skills in CLE.

We want to acknowledge potential limitations of our study. Firstly, the images were not selected and subsequently blinded for interpretation. This was because we wanted to extrapolate or replicate a real time endomicroscopy scenario whereby onsite interpretation and diagnosis was done live by the endomicroscopist with further biopsies sent to the pathologist. Nevertheless, the final histopathology reports were blinded till the end of the study of the study for final image reporting comparison and analysis of reporting groups. Secondly, we did not analyze differences in different criteria based on glandular structures or microvasculature of lesions as we wanted the interpretation to be simple as benign *vs* dysplastic/neoplastic, also the fact there is a variation in interpretation between grades of dysplasia and neoplasia for routine histopathological samples. Thirdly, we did not aim to look at differences between the endomicroscopists (*i.e.*, interobserver agreement) rather compare this group with a pathologist as we recognized each had a different role and approach to interpretation and therefore were interested in bringing this out in this study. This is the topic for further, ongoing studies. Also we acknowledge there was heterogeneity in the lesions that were studied, however it seem to reflect the pCLE application in real time clinical practice. Lastly we included one single pathologist for the study and one might argue that the accuracy interpretation results might be improved with more expert GI pathologists. We acknowledge this as a major limitation as assessment made by a single pathologist without a consolidated experience may be affected by subjective elements and therefore, the data has little value as indicator and cannot be generalized.

In summary, from our study we are able to show that reporting can be done substantially between both the gastroenterologist and the pathologist. However, there seems to be discrepancy in the interpretation of pCLE findings between them particularly for benign and pancreaticobiliary lesions. Given the unique roles of both the endomicroscopist and the pathologist, it will be interesting to see if combining them helps in improving the overall accuracy of pCLE interpretation. Further studies are needed to identify how well endomicoscopic and histopathological criteria can be molded together by merging both distinguishable features. This will be relevant especially in situations when results are indeterminate or the degree of dysplasia *vs* neoplasia is unclear.

COMMENTS

Background

Endoscopic tissue sampling with histopathology is considered the gold standard for diagnosis and management of most gastrointestinal (GI) disorders. Even though random biopsies are considered the norm, they are also involved in flaws such as sampling errors along with an incremental cost that may be incurred.

Research frontiers

Newer imaging technology such as the confocal laser endoscopy (CLE) has been developed to obtain high resolution images of the gastrointestinal mucosa allowing *in vivo* and real time endomicroscopic analysis of the targeted tissue.

Related publications

Studies have shown that CLE can be performed and interpreted accurately after adequate training. However, it is still not clear what the learning curve for adequate diagnosis and interpretation using this new technology will be in predicting better outcomes. Understanding this will have long term effects on operating costs while enhancing the benefit to the patients.

Innovations and breakthroughs

This paper aims to compare the interpretation of CLE findings between endoscopists and GI pathologists, in order to achieve a better diagnostic reproducibility and confirmation of interpretation of *in vivo* images in situations where results are indeterminate or the degree of dysplasia *vs* neoplasia is unclear. The study is broad and covers much different pathology, not choosing to focus on specific pathologic locations or lesions but assessing the sensitivity, specificity and accuracy of confocal microscopy for endoscopists and pathologists, then comparing those rates. Endomicroscopy probe-based CLE (pCLE) procedures were undertaken and the endoscopist rendered assessment. And then images viewed offline by a GI pathologist. Using histopathology as a gold standard for definitive diagnosis the study showed that the endoscopist was able to better define the nature of the lesion in real time compared with the pathologist.

Applications

The study brings to light the "real world" differences one might encounter with the interpretation of images using the confocal laser pCLE technology if the pathologist is not present in the room during the procedure. This is an initial study and further, larger studies are needed to clarify the sensitivity and specificity of this technology in specific lesions and pathologies based upon the results.

Terminology

CLE is based on tissue reflectance or tissue fluorescence after application of fluorescence agents (*e.g.*, fluorescein sodium) generating images that demonstrate cellular architecture and microvasculature that is comparable with traditional histology. The pCLE is the device consisting of several fiber light bundles (> 10000 optical fibers) with distal lens through which the laser beam is transmitted while being connected to a laser-scanning unit and light source. This is passed through the working channel of the endoscope and can be approximated to the targeted mucosal area in the gastrointestinal tract for further visualization.

Peer review

The authors have conducted a well thought out study bringing to light the "real world" differences one might encounter with the interpretation of images using the confocal laser pCLE. The topic is of potential interest in clinical practice to define and improve the outcome in endoscopic diagnosis. The paper is only focused on the analysis of the differences in interpretation by the two specialists. Although the study is planned so correct enough, it contains limitations that make the paper a pilot study.

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META-ANALYSIS

Efficacy and safety of gemcitabine-based chemotherapies in biliary tract cancer: A meta-analysis

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Abstract

AIM: To investigate the efficacy and safety of gemcitabine (Gem)-based combination chemotherapies for the treatment of advanced biliary tract cancer.

METHODS: Clinical trials were identified by searching scientific literature databases (PubMed, EMBASE and the Cochrane Library) for studies published between 1975 and 2013. Two reviewers independently evaluated the relevant studies and manually searched references from these reports to locate additional eligible studies. The disease response and control rates, progression-free and overall survivals, and the grade 3-4 toxicities were evaluated by a meta-analysis. Odds-ratios (ORs) of the disease response and control rates and grade 3-4 toxicities, and the mean difference (MD) of both progression-free and overall survivals were calculated and used for statistical analysis.

RESULTS: Seven randomized trials with a total of 858 patients were selected and included in the final analysis. The studies were divided into subgroups based on the chemotherapy regimens, including Gem-based and non-Gem-based chemotherapies. The overall analyses revealed that the patients treated with Gem-based combination chemotherapy had significantly higher disease response rates [OR = 1.69, 95% confidence interval (CI): 1.17-2.43; P = 0.01], a longer progression-free survival (MD = 1.95, 95%CI: 0.90-3.00; P = 0.00) and a longer overall survival (MD = 1.85, 95%CI: 0.26-3.44; P = 0.02). A higher incidence of grade 3-4 hematological toxicities, including leukopenia (OR = 2.98, 95%CI: 1.44-6.20; P = 0.00), anemia (OR = 2.96, 95%CI: 1.79-4.92; P = 0.00) and neutropenia (OR = 2.80, 95%CI: 1.39-5.64; P = 0.00) was found in the Gem-based combination chemotherapy group compared with the Gem monotherapy and non-Gem-based chemotherapy groups.

CONCLUSION: Gem-based combination chemotherapy is a potential first-line treatment for advanced biliary tract cancer as a result of improved survival, though with additional toxicity.

Key words: Biliary tract cancer; Combination chemotherapy; Gemcitabine; Meta-analysis; Randomized trial

Core tip: To investigate the efficacy and safety of gemcitabine (Gem)-based combination chemotherapy for the treatment of advanced biliary tract cancer, the authors analyzed the potential impact of Gem-based combination chemotherapy and other regimens on the outcomes and toxicities of the patients using meta-analysis methodologies. Meta-analysis showed that compared with Gem monotherapy and non-Gem-based chemotherapy, Gem-based combination chemotherapy provided a modest improvement in survival but was associated with more toxicity.

Liu H, Zhang QD, Li ZH, Zhang QQ, Lu LG. Efficacy and safety of gemcitabine-based chemotherapies in biliary tract cancer: A meta-analysis. *World J Gastroenterol* 2014; 20(47): 18001-18012 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/18001.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.18001

INTRODUCTION

Biliary tract cancer (BTC) refers to tumors that develop in the bile ducts and gall bladder and includes cholangiocarcinomas and gallbladder carcinomas[1]. BTC is a heterogeneous group of relatively rare tumors that account for about 3% of all gastrointestinal malignancies[1] and is the second most common cause of primary hepatic tumors[2]. Globally, hepatobiliary malignancies account for 13% of cancer-related deaths; 10%-20% of these deaths are attributable to BTC[3]. Epidemiologic studies have indicated that the incidence of BTC has increased rapidly worldwide in previous decades, particularly in Asian countries[1,4,5]. Despite advances in the diagnosis, staging and surgical management of BTC during the past decade, patients with BTC have a reported five-year survival rate that approaches only 15%[4] and an overall median survival of only 6.3 mo[6]. Surgical resection may be the only potentially curative therapeutic option. Unfortunately, due to the late clinical presentation, most BTC patients are diagnosed at an advanced stage when surgical resection is not feasible and treatment options are limited[5]. Hence, chemotherapeutic treatment is usually recommended for patients with unresectable advanced BTC or for patients who relapse subsequent to surgery[7].

Gemcitabine (Gem) emerged as a treatment for pancreatic cancer and has been explored as a treatment for advanced biliary cancer since 1998[8]. To improve the clinical efficacy, systemically administered Gem is often combined with a second cytotoxic agent, such as platinum analogs, fluoropyrimidine, or a targeted cytotoxic agent. The results from several phase Ⅱ studies suggest that Gem, alone or in combination with other agents, has been relatively effective for treating BTC[5,9]. However, most of these studies were small, single-arm and nonrandomized trials. Therefore, the role of Gem-based chemotherapy for patients with advanced BTC has not been clearly established. Until 2010, data from the largest randomized biliary tract trial to date indicated that the overall survival was significantly higher in the Gem and cisplatin arms *vs* the Gem single-agent treatment (11.7 mo *vs* 8.1 mo)[10]. Based on these results, Gem combined with cisplatin was established as the new standard of therapy for advanced, unresectable BTC[7]. Since that time, several randomized trials[11-14] comparing Gem-based combination chemotherapy with other regimens have been published. However, the results of these trials were conflicting, which has made the role of Gem-based combination chemotherapy controversial. Using these data, we conducted a meta-analysis to evaluate the efficacy and safety of Gem-based combination chemotherapy in advanced BTC treatment. The aim of this study was to assess whether Gem-based combination chemotherapy improves BTC prognosis compared with other treatment regimens.

MATERIALS AND METHODS

Search methods

PubMed, EMBASE and the Cochrane Library were systematically searched using the following combination of search terms: "biliary tract cancer", "gallbladder carcinoma", "cholangiocarcinoma" and "gemcitabine". The search was performed in August 2013 and updated in November 2013 to identify relevant publications between 1975 and 2013; there were no language restrictions. All potentially relevant studies were retrieved, and their references were evaluated to identify additional eligible studies.

Inclusion and exclusion criteria

Studies were eligible for inclusion in the meta-analysis if they met all of the following criteria: (1) advanced BTC patients (with unresectable or metastatic cancer); (2) Gem-based combination chemotherapy at any line; (3) reported disease response rate (DRR), disease control rate (DCR), progression-free survival (PFS), overall survival (OS) and toxicities; and (4) structured as randomized controlled trials. Non-randomized trials and studies that repeated existing research were excluded to avoid clinical heterogeneity between studies.

Date extraction and quality assessment

Two independent reviewers (Liu H and Zhang QD) extracted the data from the eligible studies. A third reviewer (Lu LG) was consulted to resolve any disagreements. The following data were extracted from the included studies: first author's name, year of publication, number of patients enrolled in each treatment group, patient age (median and range), proportion of male participants, treatment regimens, numbers and rates of DRR, DCR, PFS, and OS and numbers and rates of each type of grade 3-4 toxicity.

Study quality was assessed with Jadad scores[15] using the following criteria: quality of randomization, quality of allocation concealment, quality of double-blinding, and quality of withdrawals and dropouts in the study description. The studies scored one point for each criterion met. Additional points were given for each of the following conditions that were met: randomization sequence method was described by computer or randomized number, method of allocation concealment was described and was appropriate, or a detailed description of proper double blinding methods was provided. Based on these criteria, high-quality studies scored a total of at least four points.

Statistical analysis

Pooled odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for the DRR and the DCR using

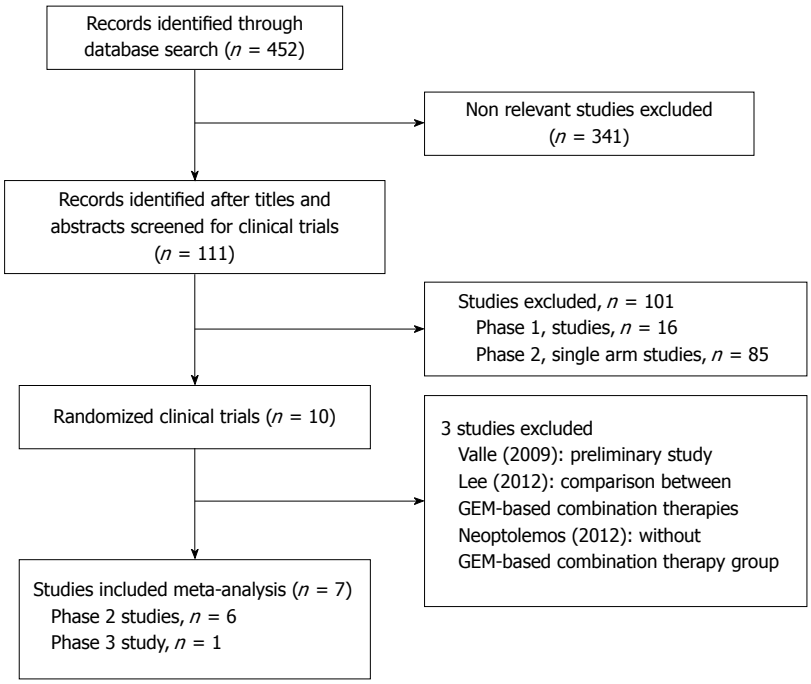

Figure 1 Flow diagram of the literature search and the study selection process. GEM: Gemcitabine.

Review Manger 5.2 software (Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). The mean differences (MDs) with 95%CIs were calculated for the DRR and the DCR. Statistical heterogeneity among studies was assessed using Cochran's Q-test and the I^2 statistic[16]. A P value < 0.10 for the Q-test or an I^2 > 50% indicated study heterogeneity; in either case, a DerSimonian-Laird random effects model was used. Otherwise, a Mantel-Haenszel fixed effects method was used. If heterogeneity was found, a sensitivity analysis was performed to identify the potential sources of heterogeneity. Egger's and Begg's tests were used to measure potential publication bias. Statistical significance from two-sided analyses was indicated by a $P < 0.05$. Subgroup analyses were used to compare the efficacy and safety of gemcitabine-based combination chemotherapy with Gem monotherapy and non-Gem-based chemotherapy.

RESULTS

Literature search and selection flow

The study selection flow is depicted in Figure 1. Initially, 452 records were identified from PubMed, EMBASE and the Cochrane Library. After the initial review of the titles and abstracts, 111 relevant clinical trials were retrieved for detailed assessment, including ten randomized trials. As the scheme depicts, seven studies[10-14,17,18] met our inclusion criteria and were included in the quantitative synthesis, while the other three studies[19-21] were excluded.

Characteristics and quality of selected studies

Table 1 summarizes the characteristics of the included studies. Four different Gem-combination chemotherapy regimens were involved in the studies: Gem and cisplatin[10,11,18], Gem and S-1[12,13], Gem and oxaliplatin[17] and Gem and mitomycin C[14]. The effects of Gem-combination chemotherapy were compared with Gem monotherapy in three studies[10,12,18] and with other chemotherapy regimens in the remaining four studies[11,13,14,17]. The seven trials provided data from a total of 858 patients (mean: 123; range: 51 to 410). Males accounted for 17.9%-66% of all subjects, and the median patient age ranged from 47 to 75 years. All patients enrolled in the included studies had the following characteristics: histologically confirmed advanced local or metastatic biliary tract cancer not amenable to surgical resection; no previous chemotherapy; Eastern Cooperative Oncology Group Performance Status scores between 0 and 2; and adequate liver, renal and hematopoietic functions.

The details of grade 3-4 toxicity assessment are shown in Table 2 and include leukopenia, neutropenia, anemia, thrombocytopenia, increased alanine aminotransferase (ALT) level, nausea, vomiting, anorexia and diarrhea.

All seven eligible studies[10-14,17,18] were randomized, and two studies[13,14] described the method of randomization with definite descriptions. Allocation concealment was performed using a proprietary algorithm in one of the trials[13]. None of the trials reported double-blind procedures. Each trial included in the meta-analysis provided a detailed description of the number of and reasons for patient withdrawals and dropouts. Finally, two studies scored 4 points or above (Table 3).

DRR

The DRRs were reported in all studies and ranged from 9.4%-36.4% without significant inter-study heterogeneity (Q = 6.69, df = 6, I^2 = 10%). The pooled OR of the DRR estimated by the fixed effects model was 1.69

Table 1 Study characteristics

Ref.	Year	Treatment	*n*	Male (%)	Age mean (range), yr	DRR (%)	DCR (%)	PFS (mo)	OS (mo)
Valle *et al*[10]	2010	GEM + CIS	204	52.9	63.9 (32.8-81.9)	21.6	81.4	8.0	11.7
		GEM	206	52.4	63.2 (23.4-84.8)	15.5	71.8	5.0	8.0
Okusaka *et al*[18]	2010	GEM + CIS	41	43.9	65 (43-80)	19.5	68.3	5.8	11.2
		GEM	42	50.0	66.5 (49-78)	11.9	50.0	3.7	7.7
Sasaki *et al*[12]	2013	GEM + S-1	30	53.3	68 (47-83)	20.0	70.0	5.6	8.9
		GEM	32	66.7	75 (55-86)	9.4	62.5	4.3	9.2
Kornek *et al*[14]	2004	GEM + MMC	25	32.0	67 (44-75)	21.7	60.9	4.2	6.7
		MMC + CAPE	26	38.5	65 (45-75)	33.3	70.8	5.3	9.3
Sharma *et al*[17]	2010	GEM + OX	26	19.2	49	30.7	68.7	8.5	9.5
		FUFA	28	17.9	47	14.3	21.4	3.5	4.6
Kang *et al*[11]	2012	GEM + CIS	49	63.3	59 (32-77)	19.6	71.7	5.7	10.1
		S-1 + CIS	48	64.6	60 (36-77)	23.8	85.7	5.4	9.9
Morizane *et al*[13]	2013	GEM + S-1	50	54.0	66 (39-78)	36.4	59.1	7.1	12.5
		S-1	51	47.1	62.5 (49-79)	17.4	39.1	4.2	9.0

CAPE: Capecitabine; CIS: Cisplatin; DCR: Disease control rate; DRR: Disease response rate; FUFA: Fluorouracil-folinic acid; GEM: Gemcitabine; MMC: Mitomycin C; OS: Overall survival; OX: Oxaliplatin; PFS: Progression-free survival.

Table 2 Percentage of cases with grade 3 or 4 toxicity *n* (%)

Ref.	Treatment	Leukopenia	Neutropenia	Anemia	Thrombo-cytopenia	Increased ALT level	Nausea	Vomiting	Anorexia	Diarrhea
Valle *et al*[10]	GEM + CIS	15.7	25.3	7.6	8.6	9.6	4.0	5.1	3.0	NR
	GEM	9.5	16.6	3.0	6.5	17.1	3.5	5.5	2.5	NR
Okusaka *et al*[18]	GEM + CIS	29.3	56.1	36.6	39.0	24.4	0.0	0.0	0.0	2.4
	GEM	19.0	38.1	16.6	7.2	16.7	0.0	0.0	2.8	0.0
Sasaki *et al*[12]	GEM + S-1	33.0	33.0	10.0	7.0	3.0	3.0	0.0	3.0	0.0
	GEM	19.0	22.0	6.0	6.0	0.0	0.0	0.0	6.0	0.0
Kornek *et al*[14]	GEM + MMC	17.0	13.0	0.0	13.0	40.0	44.0	NR	NR	28.0
	MMC + CAPE	17.0	17.0	0.0	17.0	45.0	42.0	NR	NR	28.0
Sharma *et al*[17]	GEM + OX	38.5	38.5	38.5	10.0	15.4	NR	7.7	NR	NR
	FUFA	7.1	7.1	7.1	2.0	0.0	NR	7.1	NR	NR
Kang *et al*[11]	GEM + CIS	24.4	49.0	22.4	22.4	4.1	4.1	4.1	0.0	0.0
	S-1 + CIS	0.0	31.8	2.3	4.5	0.0	2.1	0.0	0.0	4.3
Morizane *et al*[13]	GEM + S-1	29.4	60.7	11.8	11.8	13.7	2.0	2.0	7.8	2.0
	S-1	2.0	4.0	4.0	4.0	12.0	4.0	0.0	6.0	6.0

ALT: Alanine aminotransferase; CAPE: Capecitabine; CIS: Cisplatin; FUFA: Fluorouracil-folinic acid; GEM: Gemcitabine; MMC: Mitomycin C; NR: No record; OX: Oxaliplatin.

Table 3 Jadad scores of the included studies

Study	Randomization	Concealed allocation	Double-blind	Complete description
Valle *et al*[10]	1	1	0	1
Okusaka *et al*[18]	1	1	0	1
Sasaki *et al*[12]	1	1	0	1
Kornek *et al*[14]	2	1	0	1
Sharm *et al*[17]	1	1	0	1
Kang *et al*[11]	1	1	0	1
Morizane *et al*[13]	2	2	0	1

(95%CI: 1.17-2.43) (Figure 2A). The overall analysis revealed that the patients treated with Gem-based combinations achieved significantly higher DRR compared with the patients who had not received this combination chemotherapy (P = 0.01). A subgroup analysis indicated that the DRR was significantly higher for the patients treated with Gem-based combination chemotherapy compared with the patients treated with Gem alone (OR = 1.97, 95%CI: 1.20-3.21; P = 0.01). The OR for the non-Gem-based chemotherapy subgroup was 1.39 (95%CI: 0.82-2.40), which was not statistically significant.

DCR

The DCR in the studies ranged from 21.4%-86.0%. Significant heterogeneity was detected between the studies (Q = 16.57, df = 6, I^2 = 64%; P = 0.01), therefore, a random effects model was used for the meta-analysis of the DCR. The total OR for the DCR was 1.65 (95%CI: 0.91-3.00; P = 0.10) (Figure 2B). The results of the subgroup analyses indicated that the patients treated with Gem-based combinations had significantly higher DCRs compared with the patients treated with Gem alone (OR = 1.82, 95%CI: 1.18-2.81; P = 0.01). The DCR of the non-Gem-based chemotherapy subgroup was lower compared with the Gem-based combination chemotherapy group (OR = 1.48, 95%CI: 0.43-5.07), but the difference was not sig-

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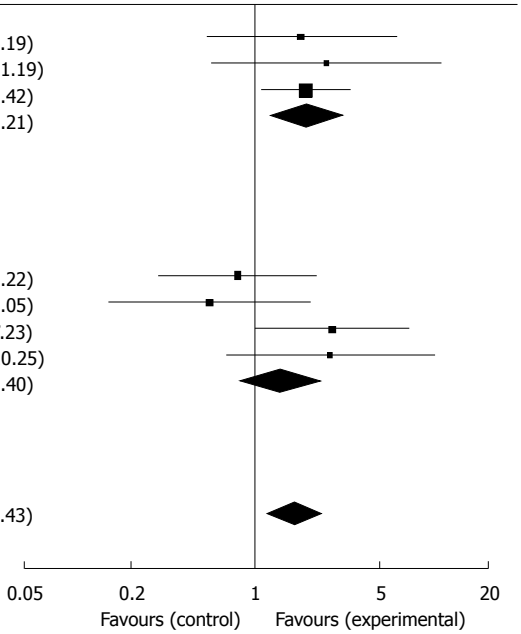
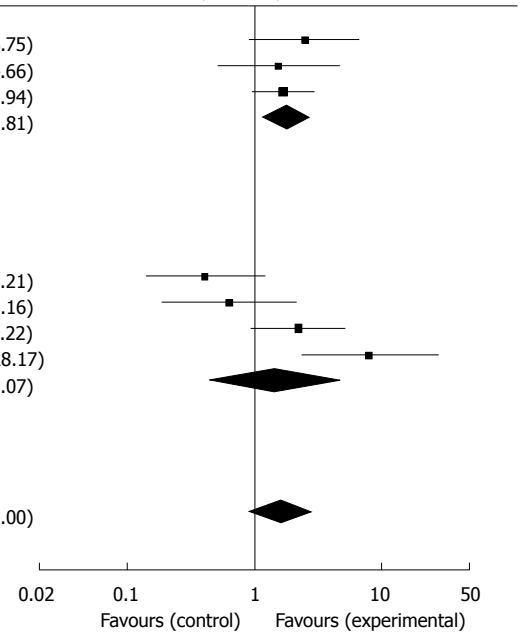
Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, fixed, 95%CI
1.1.1 Gem-based *vs* Gem						
Okusaka *et al*[18]	8	37	5	38	8.5%	1.82 (0.54, 6.19)
Sasaki *et al*[12]	6	29	3	32	5.0%	2.52 (0.57, 11.19)
Valle *et al*[10]	42	161	22	142	38.0%	1.93 (1.08, 3.42)
Subtotal (95%CI)		227		212	51.5%	1.97 (1.20, 3.21)
Total events	56		30			
Heterogeneity: $\chi^2 = 0.13$, $df = 2$ ($P = 0.94$); $I^2 = 0\%$						
Test for overall effect: $Z = 2.70$ ($P = 0.007$)						
1.1.2 Gem-based *vs* non-Gem-based						
Kang *et al*[11]	9	46	10	43	18.3%	0.80 (0.29, 2.22)
Kornek *et al*[14]	5	23	8	24	13.5%	0.56 (0.15, 2.05)
Morizane *et al*[13]	16	44	8	46	10.9%	2.71 (1.02, 7.23)
Sharma *et al*[17]	8	26	4	28	5.9%	2.67 (0.69, 10.25)
Subtotal (95%CI)		139		141	48.5%	1.39 (0.81, 2.40)
Total events	38		30			
Heterogeneity: $\chi^2 = 5.72$, $df = 3$ ($P = 0.13$); $I^2 = 48\%$						
Test for overall effect: $Z = 1.19$ ($P = 0.24$)						
Total (95%CI)		366		353	100.0%	1.69 (1.17, 2.43)
Total events	94		60			
Heterogeneity: $\chi^2 = 6.69$, $df = 6$ ($P = 0.35$); $I^2 = 10\%$						
Test for overall effect: $Z = 2.82$ ($P = 0.005$)						
Test for subgroup differences: $\chi^2 = 0.86$, $df = 1$ ($P = 0.35$); $I^2 = 0\%$						



B

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
1.2.1 Gem-based *vs* Gem						
Okusaka *et al*[18]	28	37	21	38	14.3%	2.52 (0.94, 6.75)
Sasaki *et al*[12]	21	29	20	32	13.2%	1.57 (0.53, 4.66)
Valle *et al*[10]	131	161	102	142	19.7%	1.71 (1.00, 2.94)
Subtotal (95%CI)		227		212	47.1%	1.82 (1.18, 2.81)
Total events	180		143			
Heterogeneity: $\text{Tau}^2 = 0.00$, $\chi^2 = 0.53$, $df = 2$ ($P = 0.77$); $I^2 = 0\%$						
Test for overall effect: $Z = 2.71$ ($P = 0.007$)						
1.2.2 Gem-based *vs* non-Gem-based						
Kang *et al*[11]	33	46	37	43	13.3%	0.41 (0.14, 1.21)
Kornek *et al*[14]	14	23	17	24	11.9%	0.64 (0.19, 2.16)
Morizane *et al*[13]	26	44	18	46	16.0%	2.25 (0.97, 5.22)
Sharma *et al*[17]	18	26	6	28	11.7%	8.25 (2.42, 28.17)
Subtotal (95%CI)		139		141	52.9%	1.48 (0.43, 5.07)
Total events	91		78			
Heterogeneity: $\text{Tau}^2 = 1.27$, $\chi^2 = 15.72$, $df = 3$ ($P = 0.001$); $I^2 = 81\%$						
Test for overall effect: $Z = 0.62$ ($P = 0.53$)						
Total (95%CI)		366		353	100.0%	1.65 (0.91, 3.00)
Total events	271		221			
Heterogeneity: $\text{Tau}^2 = 0.39$, $\chi^2 = 16.57$, $df = 6$ ($P = 0.01$); $I^2 = 64\%$						
Test for overall effect: $Z = 1.66$ ($P = 0.10$)						
Test for subgroup differences: $\chi^2 = 0.10$, $df = 1$ ($P = 0.76$); $I^2 = 0\%$						



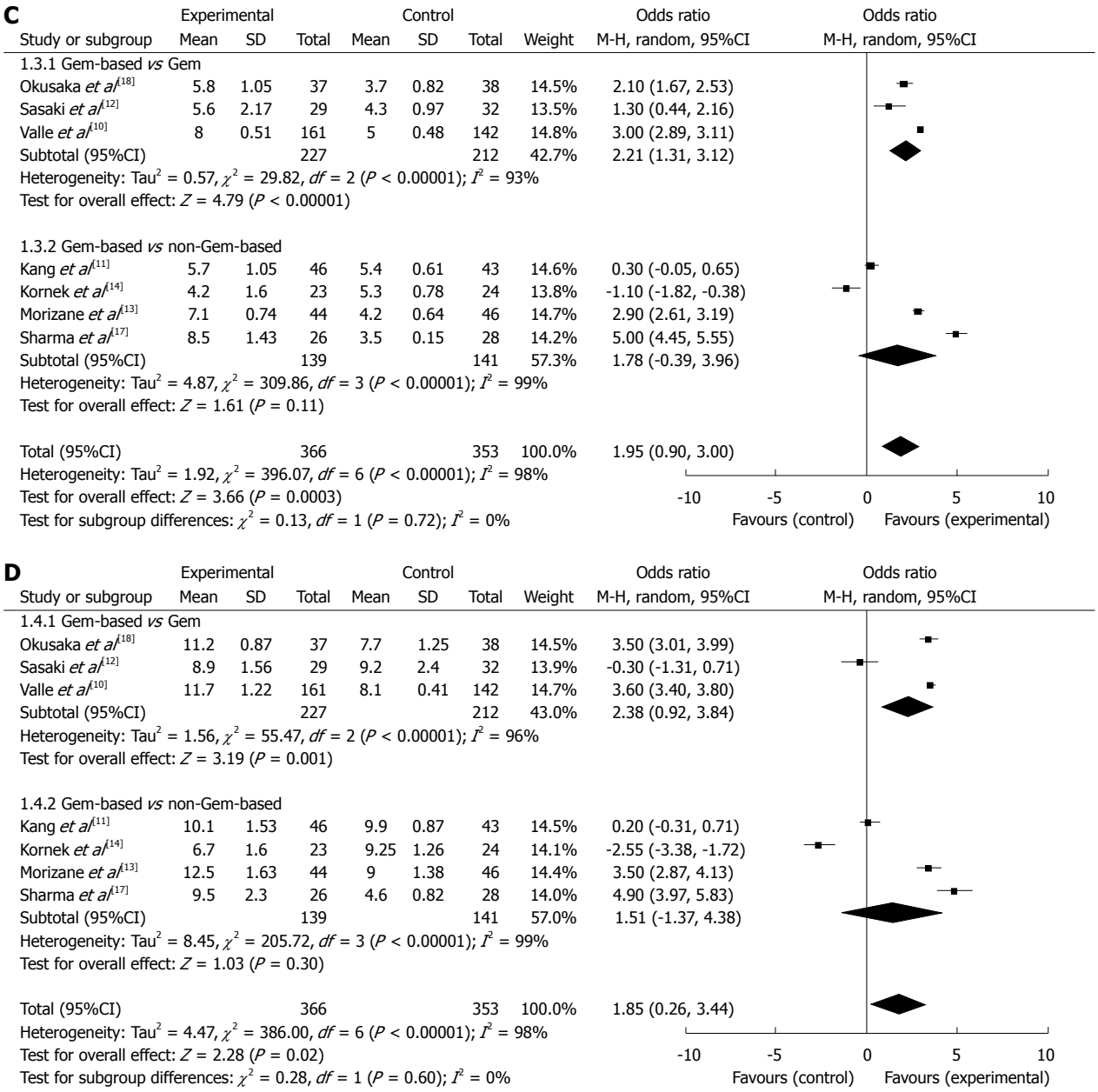


Figure 2 Forest plots of the effects of gemcitabine-based combination chemotherapy in patients with biliary tract cancer. A: Disease response rate; B: Disease control rate; C: Progression-free survival; D: Overall survival. Gem: Gemcitabine monotherapy; Gem-based: Gem-based combination chemotherapy; non-Gem-based: non-Gemcitabine-based chemotherapy.

nificant.

PFS

The median PFS of the included studies ranged from 3.5-8.5 mo. After data pooling, significant heterogeneity was observed among the studies (Q = 396.07, df = 6, I^2 = 98%; P = 0.00), therefore, a random effects model was used for the meta-analysis of the median PFS. The overall MD for the PFS was 1.95 (95%CI: 0.90-3.00; P = 0.00) (Figure 2C), which suggests that Gem-based combination chemotherapy significantly improved patient PFS. A subgroup analysis also revealed that the PFS was significantly longer for the patients treated with Gem-based combination chemotherapy compared with the patients treated with Gem alone (MD = 2.21, 95%CI: 1.31-3.12; P = 0.00). The MD for Gem-based *vs* non-Gem-based chemotherapy was 1.78 (95%CI: -0.39-3.96), but was not significantly different.

OS

The median OS ranged from 4.6-12.5 mo. Significant heterogeneity was detected between the studies (Q = 386.00, df = 6, I^2 = 98%; P = 0.00), therefore, a random effects model was used for the meta-analysis of OS. The overall MD for the OS was 1.85 (95%CI: 0.26-3.44; P = 0.02) (Figure 2D), which indicated that Gem-based combination chemotherapy significantly prolonged the overall survival time. A subgroup analysis revealed that the OS

was significantly longer for the patients treated with Gem-based combination therapy compared with the patients treated with Gem alone (MD = 2.38, 95%CI: 0.92-3.84; $P = 0.00$). The OS of the Gem-based combination chemotherapy group was longer compared with the non-Gem-based chemotherapy group (MD = 1.51, 95%CI: -1.37-4.38), but the difference was not significant.

Toxicities

The results of the meta-analysis of the main toxicities are presented in Figure 3. The ORs for grade 3-4 hematological toxicities analyzed in this study were higher in the Gem-based combination chemotherapy group compared with the Gem monotherapy and non-Gem-based chemotherapy groups. The incidence of leukopenia (OR = 2.98, 95%CI: 1.44-6.20; $P = 0.00$), anemia (OR = 2.96, 95%CI: 1.79-4.92; $P = 0.00$) and neutropenia (OR = 2.80, 95%CI: 1.39-5.64; $P = 0.00$) were all significantly different between the treatment groups, whereas no significant difference was noted for thrombocytopenia (OR = 1.71; 95%CI: 0.75-3.89). No significant difference in the increased ALT level (OR = 0.87; 95%CI: 0.58-1.30) was found between the treatment groups.

In the subgroup analysis, the ORs were 1.82-7.01 for leukopenia, 1.96-7.04 for anemia, 1.78-4.63 for neutropenia, 1.13-2.79 for thrombocytopenia, and 0.76-1.11 for the increased ALT level. There were significant differences between the Gem-based combination chemotherapy group and the Gem monotherapy group in leukopenia ($P = 0.01$), anemia ($P = 0.03$) and neutropenia ($P = 0.01$), but not thrombocytopenia or the increased ALT level. The ORs of leukopenia (OR = 7.17; $P = 0.02$) and anemia (OR = 7.04; $P = 0.00$) in the Gem-based combination chemotherapy group were over seven times greater than those in the non-Gem-based chemotherapy subgroup; no significant differences were observed for the other toxicities.

Sources of heterogeneity and sensitivity analysis

Because significant heterogeneity was detected among the studies for the DCR, PFS and OS, we performed a sensitivity analysis to explore the heterogeneity by omitting one study at a time and calculating pooled ORs for the remainder of the studies. The study by Kang *et al*[11] may have been the key contributor to the inter-study heterogeneity in the DCR meta-analysis. No heterogeneity was observed after excluding this study ($Q = 9.26$, $df = 5$, $I^2 = 46\%$; $P = 0.10$); the pooled OR for the DCR was 2.03 (95%CI: 1.21-3.40). The direction and magnitude of the pooled OR for the PFS and the pooled MD for the OS did not vary markedly with the removal of any study, which indicated good reliability (data not shown).

Publication bias

Egger's test was used to investigate potential publication bias; there was no evidence of bias for the DRR ($P = 1.00$), DCR ($P = 0.23$), PFS ($P = 0.55$), or OS ($P = 1.00$). Additionally, publication bias was not indicated by a Begg's funnel plot (Figure 4).

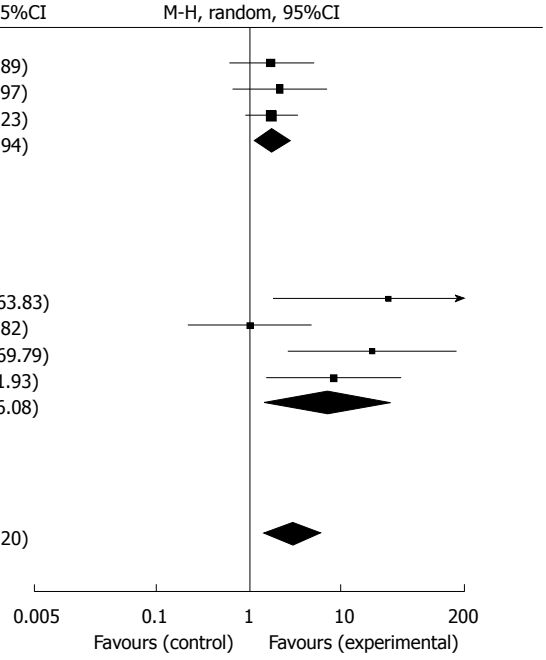
DISCUSSION

BTC is a heterogeneous group of relatively rare tumors, which often have extremely poor prognoses. Most patients present with locally advanced or metastatic disease and are candidates for surgical resection; as a result, many patients must rely on palliative chemotherapy as the only treatment option. During the last decade, several randomized controlled clinical trials have evaluated gemcitabine in combination with various agents in an attempt to improve the prognosis of advanced BTC. In this meta-analysis, we systematically evaluated the efficacy and safety of Gem-based combination chemotherapy in the treatment of advanced BTC. Our findings suggest that patients treated with Gem-based combination chemotherapy may experience better survival outcomes compared with patients not treated with this combination. However, Gem-combination chemotherapy regimens were not identical in all included studies; thus, our findings must be considered in light of this limitation.

Our overall analysis revealed that the patients treated with Gem-based combination chemotherapy had a significantly greater DRR as well as a longer PFS and OS compared with the patients not treated with this combination. Heterogeneity among the studies may be a reason for the lack of statistically significant data related to the DCR. Furthermore, the subgroup analysis revealed that the treatment with Gem-based combination chemotherapy was associated with significantly better DRR, DCR, PFS and OS outcomes compared with treatment with Gem alone. Our findings are consistent with those of Eckel *et al*[22], who conducted a meta-analysis of chemotherapy trials for advanced BTC treatment and reported that Gem-based combination chemotherapy (Gem-cisplatin or Gem-oxaliplatin) was associated with the highest DRRs and tumor control rates. However, their meta-analysis was not restricted to randomized trials. Another recent meta-analysis by Yang *et al*[23] including three randomized trials indicated that the patients with advanced BTC who were treated with Gem-based combination chemotherapy (Gem plus platinum agents) experienced better survival outcomes compared with the patients not treated with this chemotherapy combination. However, the analysis involved studies that evaluated Gem plus platinum chemotherapy; thus, new treatments, such as Gem plus S-1, were not included. A review published by Serrano *et al*[24] also suggested that Gem alone or in combination with other agents showed a better response rate that correlated with the time to progression. Additionally, regimens that contained two drugs induced higher response rates compared with single agent treatments. In our study, the subgroup analysis also indicated that Gem-based combination chemotherapy provides some benefit over non-Gem-based chemotherapy, but the result was not significant. The consistency between our findings and previous analyses further supports the use of Gem-based combination chemotherapy as a

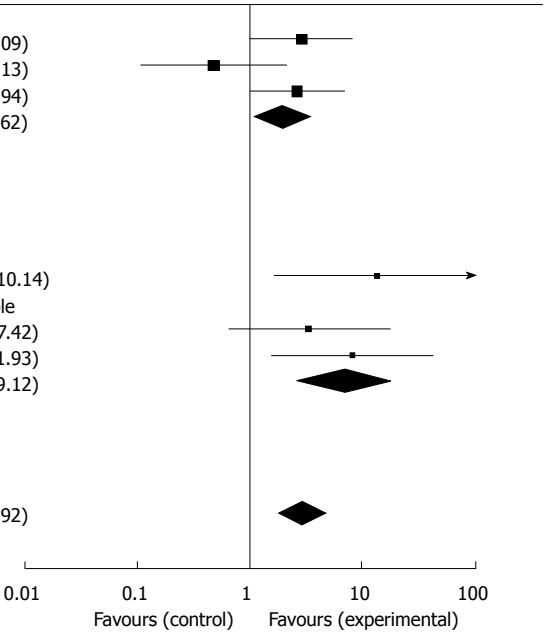
A

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
2.1.1 Gem-based *vs* Gem						
Okusaka *et al*[18]	12	41	8	42	18.9%	1.76 (0.63, 4.89)
Sasaki *et al*[12]	10	30	6	32	17.0%	2.17 (0.67, 6.97)
Valle *et al*[10]	31	198	19	199	24.9%	1.76 (1.96, 3.23)
Subtotal (95%CI)		269		273	60.8%	1.82 (1.13, 2.94)
Total events	53		33			
Heterogeneity: Tau^2 = 0.00, χ^2 = 0.10, *df* = 2 (*P* = 0.95); I^2 = 0%						
Test for overall effect: *Z* = 2.46 (*P* = 0.01)						
2.1.2 Gem-based *vs* non-Gem-based						
Kang *et al*[11]	12	49	0	48	5.4%	32.33 (1.85, 563.83)
Kornek *et al*[14]	4	23	4	24	13.1%	1.05 (0.23, 4.82)
Morizane *et al*[13]	15	50	1	51	8.8%	21.43 (2.70, 169.79)
Sharma *et al*[17]	10	26	2	28	12.0%	8.13 (1.57, 41.93)
Subtotal (95%CI)		148		151	39.2%	7.17 (1.43, 36.08)
Total events	41		7			
Heterogeneity: Tau^2 = 1.69, χ^2 = 8.32, *df* = 3 (*P* = 0.04); I^2 = 64%						
Test for overall effect: *Z* = 2.39 (*P* = 0.02)						
Total (95%CI)		417		424	100.0%	2.98 (1.44, 6.20)
Total events	94		40			
Heterogeneity: Tau^2 = 0.46, χ^2 = 12.94, *df* = 6 (*P* = 0.04); I^2 = 54%						
Test for overall effect: *Z* = 2.93 (*P* = 0.003)						
Test for subgroup differences: χ^2 = 2.54, *df* = 1 (*P* = 0.11); I^2 = 60.7%						



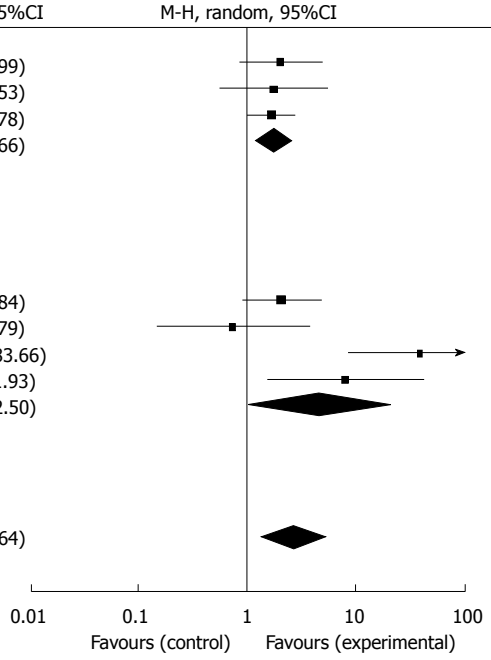
B

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, fixed, 95%CI
2.2.1 Gem-based *vs* Gem						
Okusaka *et al*[18]	15	41	7	42	23.3%	2.88 (1.03, 8.09)
Sasaki *et al*[12]	3	30	6	32	27.7%	0.48 (0.11, 2.13)
Valle *et al*[10]	15	198	6	199	29.3%	2.64 (1.00, 6.94)
Subtotal (95%CI)		269		273	80.3%	1.96 (1.07, 3.62)
Total events	33		19			
Heterogeneity: χ^2 = 4.32, *df* = 2 (*P* = 0.12); I^2 = 54%						
Test for overall effect: *Z* = 2.17 (*P* = 0.03)						
2.2.2 Gem-based *vs* non-Gem-based						
Kang *et al*[11]	11	49	1	48	4.2%	13.61 (1.68, 110.14)
Kornek *et al*[14]	0	23	0	24		Not estimable
Morizane *et al*[13]	6	50	2	51	9.2%	3.34 (0.64, 17.42)
Sharma *et al*[17]	10	26	2	28	6.3%	8.13 (1.57, 41.93)
Subtotal (95%CI)		148		151	19.7%	7.04 (2.59, 19.12)
Total events	27		5			
Heterogeneity: χ^2 = 1.19, *df* = 2 (*P* = 0.55); I^2 = 0%						
Test for overall effect: *Z* = 3.83 (*P* = 0.00001)						
Total (95%CI)		417		424	100.0%	2.96 (1.79, 4.92)
Total events	60		24			
Heterogeneity: χ^2 = 9.31, *df* = 5 (*P* = 0.10); I^2 = 46%						
Test for overall effect: *Z* = 4.21 (*P* < 0.0001)						
Test for subgroup differences: χ^2 = 4.56, *df* = 1 (*P* = 0.03); I^2 = 78.1%						



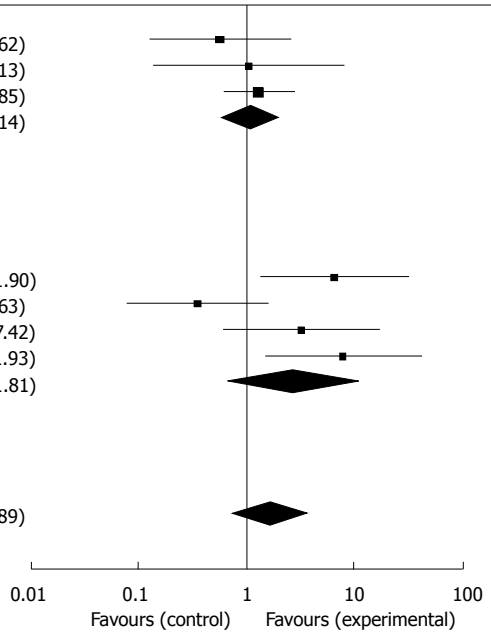
C

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
2.3.1 Gem-based *vs* Gem						
Okusaka *et al*[18]	23	41	16	42	16.8%	2.08 (0.86, 4.99)
Sasaki *et al*[12]	10	30	7	32	14.3%	1.79 (0.58, 5.53)
Valle *et al*[10]	50	198	33	199	20.4%	1.70 (1.04, 2.78)
Subtotal (95%CI)		269		273	51.4%	1.78 (1.19, 2.66)
Total events	83		56			
Heterogeneity: Tau^2 = 0.00, χ^2 = 0.15, *df* = 2 (*P* = 0.93); I^2 = 0%						
Test for overall effect: *Z* = 2.83 (*P* = 0.005)						
2.3.2 Gem-based *vs* non-Gem-based						
Kang *et al*[11]	24	49	15	48	17.2%	2.11 (0.92, 4.84)
Kornek *et al*[14]	3	23	4	24	10.3%	0.75 (0.15, 3.79)
Morizane *et al*[13]	31	50	2	51	11.0%	39.97 (8.70, 183.66)
Sharma *et al*[17]	10	26	2	28	10.1%	8.13 (1.57, 41.93)
Subtotal (95%CI)		148		151	48.6%	4.63 (0.95, 22.50)
Total events	68		23			
Heterogeneity: Tau^2 = 2.08, χ^2 = 16.26, *df* = 3 (*P* = 0.001); I^2 = 82%						
Test for overall effect: *Z* = 1.90 (*P* = 0.06)						
Total (95%CI)		417		424	100.0%	2.80 (1.39, 5.64)
Total events	151		79			
Heterogeneity: Tau^2 = 0.57, χ^2 = 19.83, *df* = 6 (*P* = 0.003); I^2 = 70%						
Test for overall effect: *Z* = 2.87 (*P* = 0.004)						
Test for subgroup differences: χ^2 = 1.31, *df* = 1 (*P* = 0.25); I^2 = 23.7%						



D

Study or subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
2.4.1 Gem-based *vs* Gem						
Okusaka *et al*[18]	3	41	5	42	14.2%	0.58 (0.13, 2.62)
Sasaki *et al*[12]	2	30	2	32	10.2%	1.07 (0.14, 8.13)
Valle *et al*[10]	17	198	13	199	22.0%	1.34 (0.63, 2.85)
Subtotal (95%CI)		269		273	46.4%	1.13 (0.60, 2.14)
Total events	22		20			
Heterogeneity: Tau^2 = 0.00, χ^2 = 0.95, *df* = 2 (*P* = 0.62); I^2 = 0%						
Test for overall effect: *Z* = 0.38 (*P* = 0.71)						
2.4.2 Gem-based *vs* non-Gem-based						
Kang *et al*[11]	11	49	2	48	13.6%	6.66 (1.39, 31.90)
Kornek *et al*[14]	3	23	7	24	14.2%	0.36 (0.08, 1.63)
Morizane *et al*[13]	6	50	2	51	12.9%	3.34 (0.64, 17.42)
Sharma *et al*[17]	10	26	2	28	13.0%	8.13 (1.57, 41.93)
Subtotal (95%CI)		148		151	53.6%	2.79 (0.66, 11.81)
Total events	30		13			
Heterogeneity: Tau^2 = 1.51, χ^2 = 9.93, *df* = 3 (*P* = 0.02); I^2 = 70%						
Test for overall effect: *Z* = 1.39 (*P* = 0.16)						
Total (95%CI)		417		424	100.0%	1.71 (0.75, 3.89)
Total events	52		33			
Heterogeneity: Tau^2 = 0.65, χ^2 = 13.62, *df* = 6 (*P* = 0.03); I^2 = 56%						
Test for overall effect: *Z* = 1.28 (*P* = 0.20)						
Test for subgroup differences: χ^2 = 1.25, *df* = 1 (*P* = 0.26); I^2 = 20.2%						



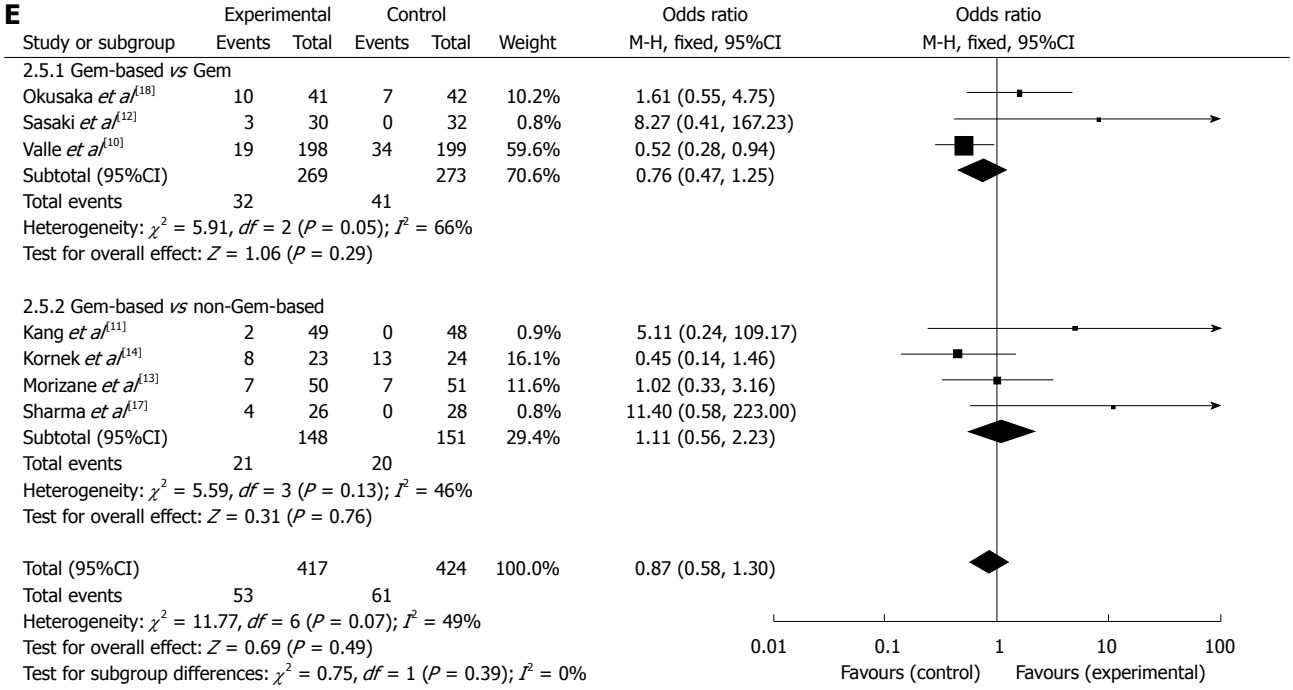


Figure 3 Forest plots of the main toxicities of gemcitabine-based combination chemotherapy in patients with biliary tract cancer. A: Leukopenia; B: Anemia; C: Neutropenia; D: Thrombocytopenia; E: Increased alanine aminotransferase level. Gem: gemcitabine monotherapy; Gem-based: Gem-based combination chemotherapy; non-Gem-based: non-Gem-based chemotherapy.

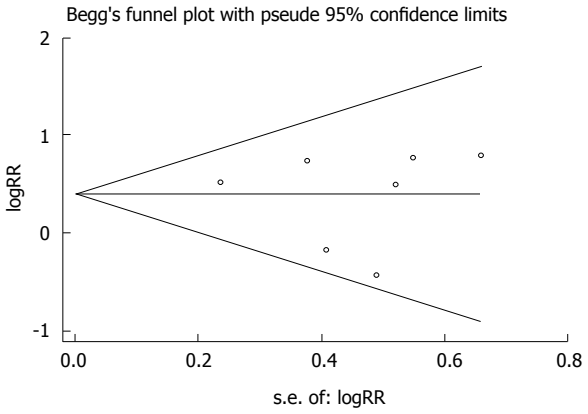


Figure 4 Begg's funnel plot for the assessment of publication bias.

first-line treatment for advanced BTC.

We also assessed the five most common toxicities related to the chemotherapeutic treatment of advanced BTC, which are leukopenia, neutropenia, anemia, thrombocytopenia and an increased ALT level. The analysis indicated that the incidence of grade 3-4 hematological toxicities, including leukopenia, neutropenia and anemia, were significantly higher following Gem-based combination chemotherapy than following Gem monotherapy or non-Gem-based chemotherapy. There was no significant difference in the rates of thrombocytopenia or the increased ALT level. Furthermore, a subgroup analysis showed that the ORs of leukopenia, neutropenia and anemia after treatment with Gem-based combination chemotherapy were almost twice those of Gem monotherapy. Additionally, the ORs of leukopenia and neutropenia in the Gem-based combination chemotherapy were more than seven times those of the non-Gem-based chemotherapy. The results presented here suggest that Gem-based combination chemotherapy induced more toxicity compared with Gem alone or non-Gem-based chemotherapy. However, all severe hematological toxicities (grade 3 or 4) are infrequent and reversible, and these results are consistent with a previous study[24].

Several limitations of our meta-analysis should be mentioned. First, only randomized trials were selected; thus, the total included sample size was small (seven studies). Additionally, our selections may have been subjected to some bias, although we observed none. Second, our data are based on the published data for which the patient outcomes according to the type of BTC were not available. This may limit our capacity to fully explore the effects of Gem-based combination chemotherapy in different types of BTC. Another limitation was that the included studies did not have homogenous characteristics with respect to the regimens used for Gem-based combination chemotherapy or the competing regimen. Furthermore, the patient demographics between the included studies were different. Due to the limitations mentioned above, the results of this meta-analysis should be interpreted with care.

In conclusion, the results of our meta-analysis of randomized trials suggest that the treatment of advanced BTC with Gem-based combination chemotherapy is associated with significantly better survival outcomes compared with treatment with Gem alone or non-Gem-based chemotherapy. Major hematological toxicities associated with Gem-based combination chemotherapy were

generally manageable and acceptable. Therefore, Gem-based combination chemotherapy should be considered as a standard first-line treatment for advanced BTC. In the future, larger multicenter randomized controlled trials should be designed to examine the efficacy and safety of Gem-based combination chemotherapy.

COMMENTS

Background

Biliary tract cancer (BTC) is a heterogeneous group of relatively rare tumors that often have extremely poor prognoses. Gemcitabine (Gem) is the treatment of choice for patients with advanced BTC. The results from several phase Ⅱ studies suggest that Gem, alone or in combination with other agents, has been relatively effective for treating BTC. However, most of these studies were small, single-arm and nonrandomized trials.

Research frontiers

The data from the largest randomized trial to date indicated that the overall survival of BTC patients was significantly higher in the Gem and cisplatin arm *vs* the Gem single-agent arm. Since that time, several randomized trials comparing Gem-based combination chemotherapy with other regimens have been published. However, the results of these trials were conflicting, which has made the role of Gem-based combination chemotherapy controversial.

Innovations and breakthroughs

Based on this meta-analysis, Gem-based combination chemotherapy was superior in disease response rate, progression-free survival and overall survival to the radiation therapy group or chemotherapy group alone. Similar results were indicated in the subgroup analyses. The Gem-based combination chemotherapy group had significantly more grade 3-4 treatment-related hematologic and non-hematologic toxicities than Gem alone or non-Gem-based chemotherapy groups. These findings were not presented clearly in previous systematic reviews.

Applications

The results of this meta-analysis of randomized trials suggest that Gem-based combination chemotherapy can improve the prognosis of patients with advanced BTC, though it may also increase the treatment-related toxicity.

Peer review

This is a well-written manuscript analyzing therapeutic management of advanced biliary tract cancer. In this manuscript, the authors compared the efficacy and safety of Gem-based combination chemotherapy with Gem alone or non-Gem-based chemotherapy. The data were collected and analyzed effectively.

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META-ANALYSIS

Meta-analysis of the efficacy of probiotics in *Helicobacter pylori* eradication therapy

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Abstract

AIM: To evaluate the role of probiotics in the standard triple *Helicobacter pylori* therapy.

METHODS: In this meta-analysis, we investigated the efficacy of probiotics in a standard triple *H. pylori* therapy in adults. Searches were mainly conducted in MEDLINE/PubMed, EMBASE, and the Cochrane Central Register of Controlled Trials. Fourteen studies met our criteria, and the quality of these studies was assessed using the Jadad scale. We used STATA version 12.0 to extract data and to calculate the odds ratios (ORs), which are presented with the corresponding 95% confidence intervals (CIs). The data are presented as forest plots.

RESULTS: The pooled ORs for the eradication rates calculated by intention-to-treat analysis and per-protocol analysis in the probiotic group *vs* the control group were 1.67 (95%CI: 1.38-2.02) and 1.68 (95%CI: 1.35-2.08), respectively, using the fixed-effects model. The sensitivity of the Asian studies was greater than that of the Caucasian studies (Asian: OR = 1.78, 95%CI: 1.40-2.26; Caucasian: OR = 1.48, 95%CI: 1.06-2.05). The pooled OR for the incidence of total adverse effects was significantly lower in the probiotic group (OR = 0.49, 95%CI: 0.26-0.94), using the random effects model, with significant heterogeneity (I^2 = 85.7%). The incidence of diarrhea was significantly reduced in the probiotic group (OR = 0.21, 95%CI: 0.06-0.74), whereas the incidence of taste disorders, metallic taste, vomiting, nausea, and epigastric pain did not differ significantly between the probiotic group and the control group.

CONCLUSION: Supplementary probiotic preparations during standard triple *H. pylori* therapy may improve the eradication rate, particularly in Asian patients, and the incidence of total adverse effects.

Key words: *Helicobacter pylori*; Eradication; Probiotics; Meta-analysis; Adult

Core tip: This systematic review and meta-analysis evaluated the role of probiotics in the standard triple *Helicobacter pylori* therapy in adults. Using a rigorous

and rational search strategy, inclusion criteria, and statistical analyses, we found that supplementary probiotic preparations given during standard triple *H. pylori* therapy conferred a higher eradication rate, particularly in Asian patients, and a lower incidence of total adverse effects, particularly diarrhea.

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INTRODUCTION

It has been more than 30 years since Australian scientists Marshall and Warren successfully cultured *Helicobacter pylori* (*H. pylori*) in 1983, and numerous studies have confirmed that *H. pylori* infection is a key risk factor for peptic ulcer, chronic atrophic gastritis, gastric cancer, and other gastrointestinal diseases. *H. pylori* is a gram-negative, microaerophilic bacterium. It is spiral in shape with a flagellum, and colonizes the human gastric mucosa. It has been estimated that 50% of the world's population could be infected with this bacterium, and in some developing countries, this number reaches 80%[1]. In most cases, bacterial colonization is present for the whole lifetime and there is a range of clinical manifestations, from asymptomatic subjects to those with serious pathologies[2,3]. Therefore, to manage those *H. pylori*-related diseases, it is important to formulate an effective *H. pylori* eradication treatment. In the past few years, the standard triple therapy, which consists of a proton pump inhibitor (PPI) and two antibiotics, is regarded as the first-line treatment[2]. The most commonly used antibiotics are tetracycline, amoxicillin, imidazole (metronidazole or tinidazol), and macrolide (clarithromycin or azithromycin). However, antibiotic-associated adverse effects, including diarrhea, nausea, vomiting, abdominal pain, and bloating, limit the use of the eradication treatment, and antibiotic resistance in *H. pylori*, especially clarithromycin resistance, affects the efficacy of the treatment[3-5]. In areas with high rates of clarithromycin resistance, the first option is a sequential or concomitant regimen[6]. The main reason for the increase in antibiotic resistance is the accumulation of point mutations in the *H. pylori* DNA, which are in most cases associated with the overuse of antibiotics[7]. Therefore, the development of a new treatment regimen that not only improves the eradication rate but also reduces the frequency of adverse effects remains the principal challenge.

Probiotics are generally considered safe microorganisms that play a crucial role in stabilizing the intragastric microecological environment. In recent years, probiotics have been used as an anti-*H. pylori* therapy. The most common microorganisms used in probiotic formulations in clinical practice include species of *Lactobacillus*, *Bifidobacterium*, *Saccharomyces*, and *Streptococcus*, as well as *Enterococcus*[8]. These may act in different ways, such as by direct competition with *H. pylori* or by improving the patients' compliance with therapy when the incidence of antibiotic-related adverse effects is reduced[9]. The inclusion of a probiotic in an *H. pylori* eradication therapy is thought to increase its efficacy or to reduce the adverse effects of the treatment. However, this remains controversial. A meta-analysis by Tong *et al*[10] suggested that supplementation with probiotics could effectively increase the eradication rate of an anti-*H. pylori* therapy and had a positive effect on *H. pylori*-therapy-related adverse effects. However, the studies examined in their meta-analysis included different treatment regimens, and it seems that not all treatment regimens have equally beneficial effects. Therefore, we performed a systematic review and meta-analysis to evaluate the role of probiotics in the standard triple *H. pylori* therapy in adults.

MATERIALS AND METHODS

Search strategy

Systematic searches were conducted independently by two investigators (Zhu R and Chen K). The searches were mainly conducted in MEDLINE/PubMed, EMBASE, and the Cochrane Central Register of Controlled Trials[11,12]. The references cited in the included articles and relevant published reports were also searched manually. The searches were confined to articles written in Chinese or English. No restriction was set on the year of publication. The latest search was updated in 2014. The following strategy was used to find eligible trials, including the keywords: "*Helicobacter pylori*" or "*H. pylori*" and "probiotic", "probiotics", "yeast", "yogurt", "*Lactobacillus*", "*Bifidobacterium*", "*Saccharomyces*", "*Enterococcus*", or "*Streptococcus*". Both free text and MeSH searches for keywords were used.

Criteria for selection

The criteria for inclusion of studies were: (1) randomized controlled trials (RCTs); (2) comprised of patients aged 18-80 years; (3) compared at least two branches of treatment consisting of a triple regimen (PPI and two antibiotics) with a placebo or no additional intervention, and the same eradication regimen plus a probiotic; and (4) primary outcome was the rate of *H. pylori* eradication, confirmed by any generally accepted method at least four weeks after treatment. The secondary outcome was the frequency of total and specific adverse effects.

Criteria for exclusion

Studies were excluded from the analysis if the loss rates were more than 20%, or if participants had suffered a chronic decompensated disease, immunological disease, or upper respiratory tract infection, or had used PPIs or H2 blockers in the preceding month. Additionally, publications that were reviews, letters, case reports, editorials, or comments were excluded.

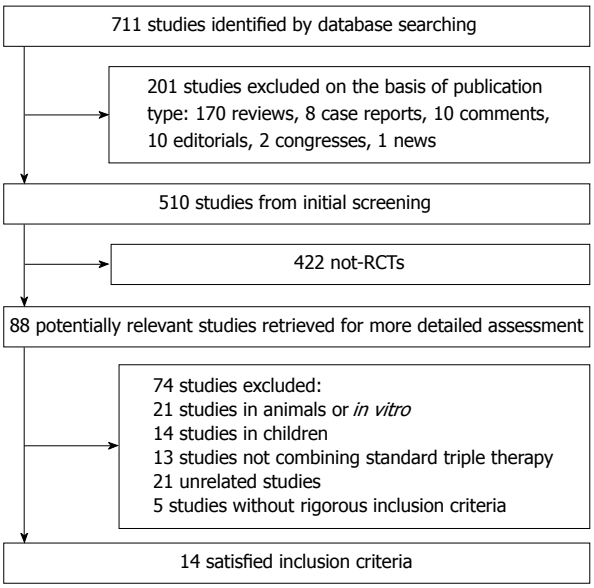

Figure 1 Study selection. RCT: Randomized controlled trial.

Selection of studies

The titles and abstracts of the studies identified by the search were read thoroughly to confirm the eligibility of the study, and the full texts of potentially eligible studies were then retrieved for further assessment. Doubts between the two investigators were discussed with a third investigator. The authors were contacted for further study details if necessary.

Assessment of methodological quality

The Jadad scale was selected to evaluate the methodological quality of eligible RCTs[13]. This scale is based on three terms: randomization (0-2 points), blinding (0-2 points), and withdrawals and dropouts (0-1 point). A score of 1 is given when randomization or blinding is mentioned, and a further point is given if they are used appropriately. A description of the number of and reasons for withdrawals and dropouts was also accorded a score of 1. The studies were considered to be of low quality when they had scores $\leqslant 2$, and of high quality for scores $\geqslant 3$[14].

Data extraction

Data were independently extracted from the full-length articles by two investigators (Zhu R and Chen K), using a predesigned form. Disagreements were resolved by discussion. The extracted information included: name of the first author, location of the trial, the number of enrolled subjects, initial/rechecking methods used to assess *H. pylori* infection, strain, the course of the probiotic treatment, the *H. pylori* eradication regimen, follow-up time, and subject loss rate. The primary outcome was the eradication rate and the secondary outcome was the incidence of total adverse effects.

Statistical analysis

All statistical analyses were performed with STATA version 12.0. Publication bias existed when a P value < 0.05 was observed. The *H. pylori* eradication rates and the incidence of adverse effects were treated as dichotomous outcomes and expressed as odds ratios (ORs). The eradication rates were analyzed with intention-to-treat (ITT) and per-protocol (PP) analyses, and the incidence of adverse effects was analyzed with an ITT analysis. Heterogeneity was investigated using the Higgins (I^2) estimate. Low heterogeneity was defined as $I^2 < 25\%$; moderate heterogeneity as $25\% < I^2 < 50\%$; and high heterogeneity as $I^2 > 50\%$. A fixed effects model was used when no heterogeneity existed and a random effects model was used to collectively analyze the accuracy indicators (Mantel and Haenszel method). The results are presented with the corresponding 95% confidence intervals (CIs) and the significance level was $\alpha = 0.05$.

RESULTS

Characteristics of the selected studies

A total of 711 studies were identified; 201 articles were excluded because they were unsuitable publication types and 422 non-RCT studies were excluded after the initial screening. Eighty-eight studies were excluded after more detailed assessments were made (21 studies were in animals or *in vitro*, 14 studies were in children, 13 studies did not use a standard triple therapy, 21 were unrelated studies, and 5 studies had no rigorous inclusion criteria), and the remaining 14 studies[15-28] were considered suitable for inclusion in the analysis. A flow diagram of the study selection process is shown in Figure 1. The initial and rechecked *H. pylori* assessments, follow-up times, loss rates, and scoring systems used to assess adverse effects are shown in Table 1. The numbers of experimental groups and context groups, the probiotic regimen, and the eradication regimens are shown in Table 2. Fourteen studies involving 2259 patients were included in the meta-analysis; 1124 patients were treated with the standard triple therapy supplemented with probiotics, and 1135 patients were treated with the standard triple therapy only or together with a placebo. The identified studies were published between 2000 and 2014. The ethnicity in five studies was Asian[17-19,23,26] and Caucasian in the remaining studies.

Publication bias

Begg's funnel plots were used to examine the publication bias and are shown in Figure 2. A P value of > 0.05 indicated that there was no evidence of substantial publication bias in the 14 studies ($z = 0.44$, Pr $> |z| = 0.661$).

Eradication rates

Data on the effects of probiotics on the *H. pylori* eradication rates were available from 14 trials (Figure 3). The pooled ORs for the eradication rates in the ITT analysis and in the PP analysis of the probiotic group *vs* the control group were 1.67 (95%CI: 1.38-2.02) and 1.68 (95%CI: 1.35-2.08), respectively, using the fixed effects model. Low heterogeneity was demonstrated between studies in both the ITT ($I^2 = 0.00\%$) and PP analyses ($I^2 = 0.00\%$).

Table 1 Initial and rechecked *Helicobacter pylori* assessments, follow-up times, loss rates, and scoring systems used to assess adverse effects in the included studies

Ref.	*H. pylori* assessment		Follow-up time	Loss rate	Side effect scoring system
	Initial	Rechecking			
Emara *et al*[15]	HpSA, RUT, Histology	HpSA, RUT, Histology	4 wk[1] 6 wk[2]	0.00%	Non-Boer
Medeiros *et al*[16]	Culture	UBT	≥ 6 wk	0.00%	Not reported
Song *et al*[17]	RUT, Histology	UBT	4 wk	8.50%	Non-Boer
Du *et al*[18]	RUT, UBT, Pathologic examination	UBT	4 wk	2.60%	Non-Boer
Deguchi *et al*[19]	Culture, Histology, RUT	UBT, HpSA	8 wk	5.20%	Not reported
Mirzaee *et al*[20]	UBT	UBT	4 wk	16.20%	Non-Boer
Canducci *et al*[21]	UBT, Histology	UBT, Histology, Endoscopy	6 wk	2.50%	By de Boer *et al*
Nista *et al*[22]	UBT	UBT	6 wk	5.70%	By de Boer *et al*
Sheu *et al*[23]	Histology, RUT	UBT, Histology, RUT	4 wk[1] 8 wk[2]	6.90%	Non-Boer
Myllyluoma *et al*[24]	Rapid whole blood test, UBT, EIA serology	UBT, EIA, serology	4 wk[1] 4 mo[2]	0.00%	By de Boer *et al*
Yaşar *et al*[25]	Histology	UBT	4 wk	8.90%	Non-Boer
Kim *et al*[26]	RUT, Histology	UBT	4-6 wk	3.00%	Non-Boer
Scaccianoce *et al*[27]	Histology	UBT	4-6 wk	3.00%	Non-Boer
Cindoruk *et al*[28]	Histology	UBT	6 wk	0.00%	By de Boer *et al*

[1]Probiotic group; [2]Control group. EIA: Enzyme immunoassay; HpSA: *H. pylori* stool antigen test; RUT: Rapid urease test; UBT: C13 or C14 urea breath test.

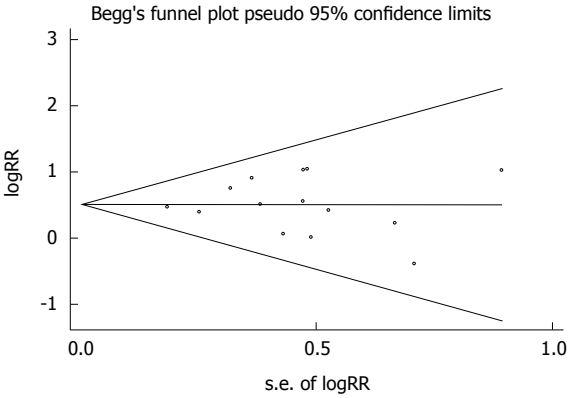

Figure 2 Funnel plot of the eradication rates in the included studies.

The overall pooled OR did not change significantly when any single study was excluded, with results ranging from 1.32 to 2.14. The following four criteria were also used to examine the stability of the analysis: (1) The removal of six poor-quality studies (Jadad score ≤ 2); (2) the removal of two studies that used combined probiotic preparations; (3) patients were divided into two categories according to ethnicity: five studies included Asian patients and nine studies included Caucasian patients; and (4) studies were divided into two categories according to the duration of triple therapy: 10 studies included a 7 d-triple therapy and four studies included triple therapy lasting more than seven days. Our results show that there was no significant difference in the pooled indices of the eight studies with Jadad scores ≥ 3, in the 12 studies that used single probiotic preparations, or when the 14 studies were included. There was also no significant difference between studies that used triple therapy regimens lasting seven days and those lasting more than seven days. These studies also had overlapping confidence intervals. However, the sensitivity of the Asian studies was greater than that of the Caucasian studies (Asian: OR = 1.78, 95%CI: 1.40-2.26; Caucasian: OR = 1.48, 95%CI: 1.06-2.05).

Adverse effects

Ten studies provided data on the incidence of total adverse effects. The pooled OR for the incidence of total adverse effects was significantly lower in the probiotic group (OR = 0.49, 95%CI: 0.26-0.94) using the random effects model due to significant heterogeneity (I^2 = 85.7%) (Figure 4A). The studies were than divided into two categories according to the probiotic strains used. Significant heterogeneity was observed in the four studies that included *Lactobacillus* and in another six studies without *Lactobacillus*. Individual adverse effects, such as taste disorders, metallic taste, diarrhea, vomiting, nausea, and epigastric pain, were also analyzed. Probiotic supplementation significantly reduced the incidence of diarrhea (OR = 0.21, 95%CI: 0.06-0.74), whereas the incidence of taste disorders (OR = 0.73, 95%CI: 0.45-1.19), metallic taste (OR = 0.87, 95%CI: 0.20-3.72), vomiting (OR = 0.40, 95%CI: 0.15-1.08), nausea (OR = 0.66, 95%CI: 0.42-1.04), and epigastric pain (OR = 0.55, 95%CI: 0.20-1.57) did not differ significantly between the probiotic group and the control group (Figures 4B, C).

DISCUSSION

As we know, *H. pylori* is closely associated with peptic ulcers, chronic atrophic gastritis, gastric cancer, and other gastrointestinal diseases. The risk of developing *H. pylori*-associated diseases may increase with increasing levels

Table 2 Numbers of experimental and context groups, and probiotic and eradication regimens

Ref. (location)	Ethnicity	Total (exp/cont)	Regimen		Jadad scores
			Probiotic	Eradication	
Emara *et al*[15] (Egypt)	Caucasian	70 (35/35)	*Lactobacillus reuteri* (DSM 17938 and ATCC PTA 6475), 2×10^8 CFU *qd* for 4 wk	O: 20 mg A: 1000 mg C 500 mg *bid* for 14 d	5
Medeiros *et al*[16] (Portugal)	Caucasian	62 (31/31)	*Lactobacillus acidophilus* (BioSaúde laboratories, Portugal), 15×10^9 CFU, 10×10^9 CFU *qn* for 8 d	E: 20 mg A: 1000 mg C: 500 mg *bid* for 8 d	2
Song *et al*[17] (Korea)	Asian	661 (330/331)	*Saccharomyces boulardii* (Bioflor250, Kuhnil Pharmacy, Seoul, Korea), 3×10^{10} CFU *tid* for 4 wk	O: 20 mg A: 1000 mg C: 500 mg *bid* for 7 d	3
Du *et al*[18] (China)	Asian	156 (77/79)	*Lactobacillus acidophilus*, 107 CFU, Streptococcus faecalis, 5×10^6 CFU, Bacillus subtilis, 104 CFU *tid* for 2 wk	O: 20 mg A: 1000 mg C: 500 mg *bid* for 7d	3
Deguchi *et al*[19] (Japan)	Asian	229 (115/114)	*Lactobacillus gasseri* (OLL2716), $\geqslant 10^9$ CFU *bid* for 4 wk	R: 10 mg A: 750 mg C: 200 mg *bid* for 7 d	3
Mirzaee *et al*[20] (Iran)	Caucasian	68 (34/34)	*Probiotic yogurt* (1.5% fat), 150 mg *bid* for 7 d	P: 40 mg *qd* A: 1000 mg *bid* C: 500 mg *bid* for 7 d	2
Canducci *et al*[21] (Italy)	Caucasian	120 (60/60)	*Lactobacillus acidophilus* strain LB, $\geqslant 5 \times 10^9$ heat-killed organisms *tid* for 10 d	R: 20 mg *bid* C: 250 mg tid A: 500 mg tid for 7d	3
Nista *et al*[22] (Italy)	Caucasian	106 (54/52)	*Bacillus clausii* (Sanofi-Synthelabo OTC, Milan, Italy), 2×10^9 CFU *tid* for 14 d	R: 20 mg A: 1000 mg C: 500 mg *bid* for 7d	4
Sheu *et al*[23] (Taiwan)	Asian	160 (80/80)	*Bifidobacterium*-containing yogurt, $\geqslant 5 \times 10^9$ live organisms per bottle *bid* for 4 wk	L: 30 mg A: 1000 mg C: 500 mg *bid* for 7 d	2
Myllyluoma *et al*[24] (Finland)	Caucasian	47 (23/24)	Probiotics (Valio Ltd, Helsinki, Finland), 65×10^9 CFU *bid* for 1 wk 65×10^9 CFU *qd* for 3 wk	L: 30 mg C: 500 mg A: 1000 mg *bid* for 7d	4
Yaşar *et al*[25] (Turkey)	Caucasian	76 (38/38)	*Bifidobacterium* (DN-173 010-10), 10^{10} CFU *qd* for 14 d	P: 40 mg A: 1000 mg C: 500 mg *bid* for 14 d	2
Kim *et al*[26] (Korea)	Asian	347 (168/179)	*Lactobacillus acidophilus* (HY 2177), $> 15 \times 10^6$ CFU, *L. casei* (HY 2743), $> 15 \times 10^6$ CFU, *B. longum* (HY 8001), $> 15 \times 10^7$ CFU, *S. thermophilus* (B-1), $> 15 \times 10^9$ CFU *qd* for 3 wk	Standard PPI C: 500 mg A: 1000 mg *bid* for 7d	2
Scaccianoce *et al*[27] (Italy)	Caucasian	33 (17/16)	*Lactobacillus reuteri* (ATCC 55730), 10^8 CFU *bid* for 7 d	L: 30 mg A: 1000 mg C: 500 mg *bid* for 7d	1
Cindoruk *et al*[28] (Turkey)	Caucasian	124 (62/62)	*Saccharomyces boulardii*, 1 gram (250 mg sachets, 500 mg *bid* Reflor (Sanofi-Synthelabo Ilac A.S., Istanbul, Turkey) *bid* for 2 wk	L: 30 mg A: 1000 mg C: 500 mg *bid* for 14 d	4

A: Amoxicillin; C: Clarithromycin; cont: Control; CFU: Colony forming units; E: Esomeprazole; exp: Experimental; L: Lansoprazole; O: Omeprazole; P: Pantoprazole; PPI: Proton pump inhibitor; R: Rabeprazole.

of *H. pylori*[29,30]. For the past few years, the standard triple therapy, as recommended by the Maastricht 2-2000 Consensus Report, is regarded as the first-line treatment. However, the Maastricht 4-2012 Consensus Report recommends sequential or concomitant regimens as the best first-line treatments in areas with high rates of clar-

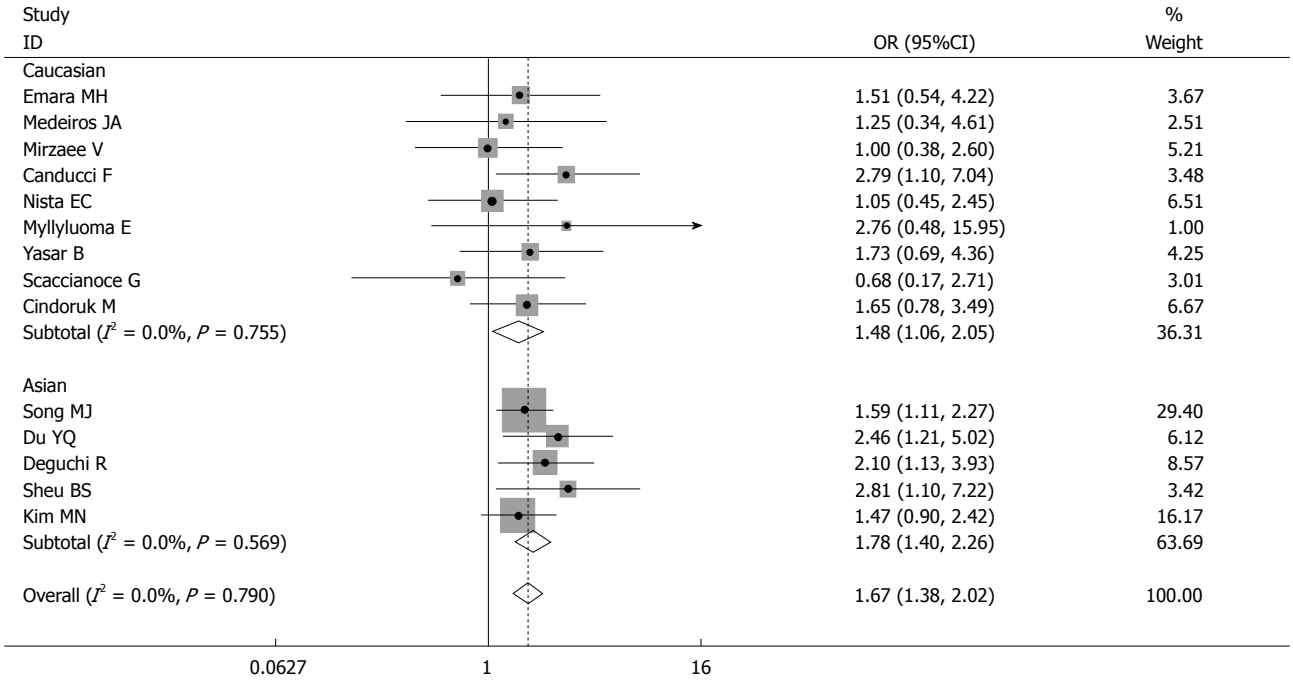

Figure 3 Meta-analysis of studies that evaluated the effects of probiotic supplementation on eradication rates by intention-to-treat.

ithromycin resistance. Other treatment regimens include quadruple therapy and miscellaneous therapy. However, unsatisfactory *H. pylori* eradication rates and antibiotic-related adverse effects remain as the two limitations of anti-*H. pylori* therapies.

A probiotic is defined as a living microbial species that may have a positive effect on the bowel microecology and improve health[31]. Currently, the most studied probiotics are lactic acid-producing bacteria, particularly *Lactobacillus* species[32]. In recent years, the use of probiotics combined with a standard triple therapy has been considered a novel choice. Probiotics may act as surrogate normal microflora after antibiotic therapy until recovery is achieved, although the mechanism is not completely understood[33]. Lesbros-Pantoflickova *et al*[34] summarized several putative mechanisms by which probiotics can inhibit *H. pylori*, including non-immunological mechanisms, antimicrobial substances, and the *in vitro* inhibitory effects of certain probiotics that are probably related to lactic acid and/or other antibacterial substances yet to be identified. Many clinical trials have suggested that probiotic supplementation is a good strategy to enhance the effectiveness of anti-*H. pylori* therapy and to reduce antibiotic-associated adverse effects, but this remains controversial. Therefore, we conducted this meta-analysis of the evidence in 14 RCTs to provide a quantitative assessment of the efficacy of probiotic supplementation in *H. pylori* eradication.

In our meta-analysis, the results of 14 RCTs pooled with a fixed effects model indicated that probiotic supplementation of a standard triple therapy regimen improved the *H. pylori* eradication rates in both ITT and PP analyses, which is consistent with eradication rates reported in a previous meta-analysis by Tong *et al*[10]. However, this result should be interpreted with care because the studies differed widely in their designs and in the antibiotic and probiotic treatments used. In a subanalysis, the *H. pylori* eradication rate was not related to the quality of the included studies, the probiotic preparations, or the duration of the triple therapy, but was greater in Asian subjects. This may be closely related to the distribution of CYP2C19 polymorphisms, which affect *H. pylori* eradication rates[35]. However, in our meta-analysis, only five studies included Asian patients, whereas nine studies included Caucasian patients, so further clinical studies are required to confirm this speculation.

The effect of probiotic supplementation on antibiotic-associated gastrointestinal adverse effects during anti-*H. pylori* regimens were also examined in our meta-analysis. The results showed that probiotics had a positive effect on the overall *H. pylori*-therapy-related adverse effects, with significant heterogeneity. Several factors may have given rise to this heterogeneity, including patient characteristics and the probiotic regimens used (species, number of colony-forming units given, duration of administration, *etc.*). Therefore, more clinical trials are required to confirm these results. From the perspective of individual adverse effects, probiotic supplementation significantly reduced the incidence of diarrhea. However, it should be noted again that the studies differed with respect to the antibiotic and probiotic treatments used, making the interpretation of the results difficult. Bühling *et al*[36] proposed that the supplementation of a PPI-antibiotic regimen with probiotics corrects antibiotic-induced intestinal dysbiosis. No study has demonstrated the complete eradication of *H. pylori* infection with probiotic treatment[37]. However, these probiotic strains can improve patient compliance by reducing antibiotic-associated adverse events, increasing the number of patients who complete

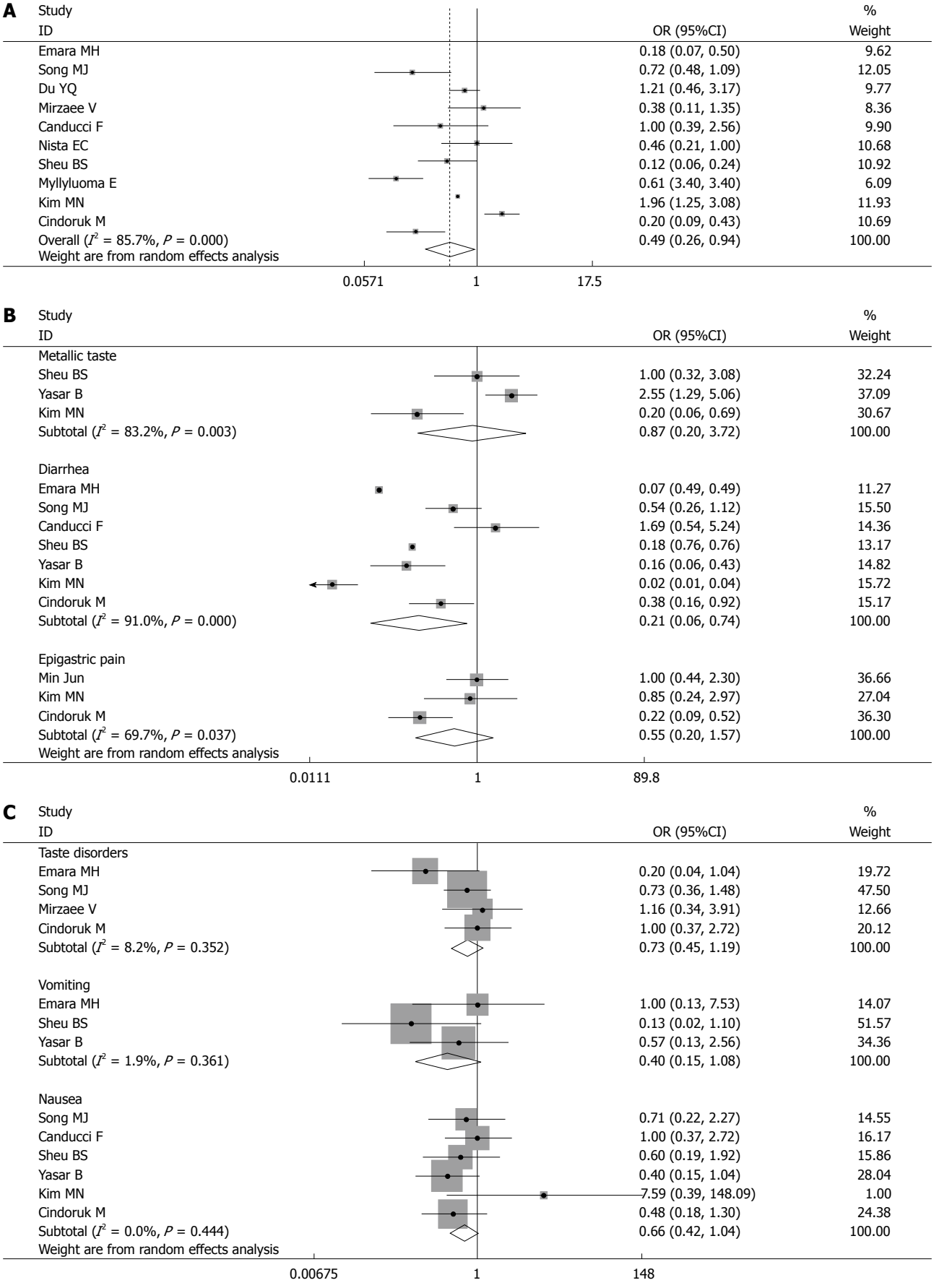

Figure 4 Meta-analysis of studies that evaluated the effects of probiotic supplementation on the incidence of adverse effects. A: Total adverse effects; B: Individual adverse effects including metallic taste, diarrhea, and epigastric pain; C: Individual adverse effects including taste disorders, vomiting and nausea.

the eradication therapy, and thus improving eradication rate.

In this study, a rigorous and rational search strategy, inclusion criteria, and statistical analyses were used to systematically and comprehensively analyze the effects of probiotics on a standard triple therapy for *H. pylori* in adults. However, this study had many limitations. First, because of the language barrier, non-English and non-Chinese studies could not be evaluated. Second, there was no standardized protocol regarding the species of probiotic, the dose, or the duration of supplementation in these studies, which will inevitably affect the results. It also seems that not all probiotics contribute equal beneficial effects. Third, there have been no trials involving patients from North America or Black individuals.

Finally, our study suggests that probiotic supplementation during *H. pylori* eradication therapy in adults may have beneficial effects on the eradication rate, particularly in Asian patients, and the incidence of total adverse effects, particularly diarrhea. More studies with rigorous designs, large sample sizes, and multiregional cooperation are required to obtain further evidence of the efficacy of probiotics in *H. pylori* eradication therapies.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is a key risk factor for many gastrointestinal diseases, such as peptic ulcers and chronic atrophic gastritis. Currently, there are no ideal treatments available that provide high eradication rates and few antibiotic-related adverse effects. The inclusion of a probiotic in *H. pylori* eradication therapy is thought to increase the efficacy or to reduce the adverse effects of the treatment. However, this remains controversial.

Research frontiers

Probiotics are safe microorganisms and stabilize the intragastric microecological environment. Several systematic reviews were recently performed to investigate the role of probiotics in *H. pylori* eradication therapies.

Innovations and breakthroughs

This meta-analysis confirms that probiotic supplementation during *H. pylori* eradication therapy improves *H. pylori* eradication rates, particularly in Asian patients, and decreases the incidence of total adverse effects.

Applications

The study results suggest that probiotic supplementation can be used in *H. pylori* eradication therapy in adults, considering the higher eradication rate and lower incidence of adverse effects.

Peer review

This is a methodologically sound meta-analysis of the probiotic effect on *H. pylori* eradication and side effects of the treatment. The statistical section is correct using updated meta-analytical methods.

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META-ANALYSIS

Three-field *vs* two-field lymph node dissection for esophageal cancer: A meta-analysis

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Abstract

AIM: To assess the effects of 3-field lymphadenectomy for esophageal carcinoma.

METHODS: We conducted a computerized literature search of the PubMed, Cochrane Controlled Trials Register, and EMBASE databases from their inception to present. Randomized controlled trials (RCTs) or observational epidemiological studies (cohort studies) that compared the survival rates and/or postoperative complications between 2-field lymphadenectomy (2FL) and 3-field lymphadenectomy (3FL) for esophageal carcinoma with R0 resection were included. Meta-analysis was conducted using published data on 3FL *vs* 2FL in esophageal carcinoma patients. End points were 1-, 3-, and 5-year overall survival rates and postoperative complications, including recurrent nerve palsy, anastomosis leak, pulmonary complications, and chylothorax. Subgroup analysis was performed on the involvement of recurrent laryngeal lymph nodes.

RESULTS: Two RCTs and 18 observational studies with over 7000 patients were included. There was a clear benefit for 3FL in the 1- (RR = 1.16; 95%CI: 1.09-1.24; $P < 0.01$), 3- (RR = 1.44; 95%CI: 1.19-1.75; $P < 0.01$), and 5-year overall survival rates (RR = 1.37; 95%CI: 1.18-1.59; $P < 0.01$). For postoperative complications, 3FL was associated with significantly more recurrent nerve palsy (RR = 1.43; 95%CI: 1.28-1.60; $P = 0.02$) and anastomosis leak (RR = 1.26; 95%CI: 1.05-1.52; $P = 0.09$). In contrast, there was no significant difference for pulmonary complications (RR = 0.93; 95%CI: 0.75-1.16, random-effects model; $P = 0.27$) or chylothorax (RR = 0.77; 95%CI: 0.32-1.85; $P = 0.69$).

CONCLUSION: This meta-analysis shows that 3FL improves overall survival rate but has more complications. Because of the high heterogeneity among outcomes, definite conclusions are difficult to draw.

Key words: Oesophagus; Cancer; Lymph node dissection; Survival; Complication

Core tip: Surgery for esophageal cancer includes removal of the primary lesion and lymph node dissection; however, there is a long-standing debate concerning the application of 3-field lymphadenectomy (3FL). The main purpose of the present meta-analysis was to present all available evidence in a systematic, quantitative, and unbiased fashion to establish the following 3 points: the effect of 3FL on the overall survival rate, identification of postoperative complications of 3FL compared to 2-field lymphadenectomy, and description of patient characteristics of those who will likely benefit from 3FL.

Ma GW, Situ DR, Ma QL, Long H, Zhang LJ, Lin P, Rong TH. Three-field *vs* two-field lymph node dissection for esophageal cancer: A meta-analysis. *World J Gastroenterol* 2014; 20(47): 18022-18030 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/18022.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.18022

INTRODUCTION

Esophageal cancer is one of the most lethal malignancies and has a long-term overall survival (OS) rate of only approximately 25% in most Western series[1], while in some Japanese series, the 5-year OS rate was reported to be approximately 50%[2]. Extensive research has been conducted to improve treatment options, especially for the optimal extent of lymph node dissection. Since 1983, several Japanese institutions[3,4] have employed radical 3-field lymphadenectomy (3FL) of the bilateral cervical, mediastinal, and abdominal regions, with the theoretical justification that relapse of cervical lymph node occurs frequently[5,6].

After almost 30 years, however, 3FL is not widely applied because its advantages and disadvantages remain controversial, resulting in an increasing amount of research focusing on identifying optimal patients and clarifying indications for 3FL recently. Nevertheless, esophagectomy remains technically demanding, and few centers can recruit a sufficient number of patients to perform clinical trials that can withstand scrutiny.

The present meta-analysis aimed to investigate the following 3 primary points: (1) the effect of 3FL on the OS rate; (2) a comparison of postoperative complications between 2FL and 3FL, and (3) identification of optimal patients who will most likely benefit from 3FL. To comprehensively and credibly answer these queries, we conducted a detailed meta-analysis using data from currently available studies that compared 3FL with 2FL, including 2 randomized controlled trials (RCTs)[7,8] and 18 observational studies[9-26]. The meta-analysis was performed on data from 1-, 3-, and 5-year OS rates, complications (recurrent nerve palsy, anastomosis leak, chylothorax, and pulmonary complications), and subgroups of recurrent laryngeal lymph node involvement. The article was arranged using a guide for reporting meta-analysis of observational studies[27].

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved by the Ethics Committee of Sun Yat-sen University Cancer Center.

Search strategy

To identify relevant studies, we conducted a computerized literature search of the PubMed, Cochrane Controlled Trials Register, and EMBASE databases from their inception to present. The search terms included the following: (1) "three-field", "3-field", "three field", "3 field", "extended cervical", "cervical lymph node dissection", "cervical lymphadenectomy", "neck lymph node dissection", "neck lymphadenectomy", "3-F", "3F", and "3FL"; (2) esophageal neoplasms (MeSH); and (3) a combination of (1) and (2).

The titles and abstracts of the identified studies were scanned to exclude any study that was clearly irrelevant. The full texts of the remaining articles were read to determine whether they contained information on the topic of interest. The reference lists of articles with relevant information were reviewed to identify citations to other studies on the same topic.

Selection criteria

The reports considered in this meta-analysis were either RCTs or observational epidemiological studies (cohort studies) that compared the survival rates and/or postoperative complications between 2FL and 3FL for esophageal carcinoma with R0 resection. There was no restriction regarding the language of articles. Articles were excluded from the analyses if they (1) contained insufficient published data for determining an estimate of relative risk (RR) or confidence interval (CI); (2) included special restrictions to types and/or stages of esophageal carcinoma (restriction to squamous carcinoma was not included because almost all tumors are of this type); (3) randomly applied 2FL or 3FL to the included patients; (4) did not apply curative resection to all included patients; and/or (5) were of poor quality and led to large biases in the analysis. In addition, for studies with multiple publications from the same population, only one with the largest data set was included. We did not assess the methodological quality of the primary studies because quality assessment in meta-analysis is controversial. Scores constructed in an ad-hoc fashion may lack demonstrated validity, and results may not be associated with quality[28].

Information extraction

Two reviewers independently extracted data using predefined criteria from each study, including the following: (1) Basic information comprising the first author's last name, year of publication, journal name, study region, study design, type and stage of esophageal tumor, and inclusion and/or exclusion criteria; (2) Published data, including the 1-, 3-, and 5-year OS rates (collected by 2 methods provided by the author or measurement of the Kaplan-Meier survival curve with the software Engauge.exe), study population, operation time, complications, and subgroup data.

When more than one estimate of effect (RR) was presented in observational studies, the most adjusted estimate was chosen. Differences in data extraction were resolved by consensus and reference to the original articles.

Statistical analysis

We performed 3 comparisons between 3FL and 2FL for esophageal carcinoma-the OS rate, complications, and subgroups. For OS, 1-, 3-, and 5-year rates were compared; for complications, recurrent nerve palsy, anastomosis leak, pulmonary complications, and chylothorax were included; and for subgroup analysis, studies with recurrent laryngeal lymph node positivity/negativity were included.

Data from each study were analyzed using Review Manager software (RevMan version 5.0; http://ims.cochrane.org/revman/download). Treatment effects were expressed as RRs with 95%CIs for dichotomous outcomes. Because mortality or morbidity was not a small probability event in the participants, the Mantel-Haenszel analysis method was used[29].

We separately pooled RR estimates from each study for each outcome using random-effects meta-analysis. Statistical heterogeneity of the RRs was evaluated using the χ^2-test with significance set at $P < 0.01$, and the I^2 statistic was calculated; publication bias was assessed using funnel plots. Low, moderate, and high degrees of heterogeneity correspond to I^2 values of 25%, 50%, and 75%, respectively. Sensitivity analyses were used to evaluate whether the results could have been markedly affected by a single study.

RESULTS

Search results

The references (n = 334) were retrieved by the original search strategy or by manual searches (n = 58). The abstracts were reviewed, and 61 articles were selected for full-text evaluation. After applying the inclusion and exclusion criteria, 20 articles were finally included (Table 1). The flow chart of the study inclusion process is shown in Figure 1.

Meta-analysis of studies on the OS rates

Twelve studies were used for 1-year OS rate analysis, including 2554 3FL patients and 3917 2FL patients. Only 5 studies reported a statistically significant difference between 2FL and 3FL, with a better OS rate in the 3FL group. Among studies with no statistical significance, 6 reported a higher 1-year OS rate in the 3FL group, whereas 1 reported a lower rate, which raised concerns regarding the significance of 3FL. Meta-analysis of all 12 studies showed a statistically significant difference between 3FL and 2FL, with a pooled RR of 1.16 (95%CI: 1.09-1.24; $P < 0.00001$; random-effects model) and statistical heterogeneity ($P = 0.0003$; $I^2 = 61\%$). 3FL showed a significant improvement in the 1-year OS rate.

The 3-year OS rate presented in 13 studies included 2598 3FL patients and 3961 2FL patients. Four studies reported a statistically significant difference with a better OS rate in the 3FL group. Studies with no statistical significance reported a higher 3-year OS rate. Meta-analysis of all 13 studies showed statistically significant differences between 3FL and 2FL with a pooled RR of 1.44 (95%CI: 1.19-1.75, $P < 0.00001$, random-effects model) and statistical heterogeneity ($P < 0.00001$; $I^2 = 82\%$). 3FL showed a significantly higher 3-year OS rate.

The 5-year OS rate reported in 12 studies included 2827 3FL patients and 4157 2FL patients. Only 3 studies reported a statistically significant difference with a better OS rate in the 3FL group. Among the 9 studies with no statistical significance, 8 reported a higher 5-year OS rate in the 3FL group, while 1 reported a lower rate. Meta-analysis of all 12 studies showed a statistically significant difference between 3FL and 2FL with a pooled RR of 1.37 (95%CI: 1.18-1.59; $P = 0.0002$; random-effects model) and statistical heterogeneity ($P < 0.00001$; $I^2 = 69\%$). 3FL showed a significant improvement in the 3-year OS rate. The forests plots are shown in Figure 2, Figure 3 and Figure 4.

Meta-analysis of studies on postoperative complications

After reviewing the postoperative complications, we included the 4 most common complications for analysis; these complications included recurrent nerve palsy, anastomosis leak, chylothorax, and pulmonary complications. The meta-analysis results from all studies demonstrated that 3FL was associated with more complications than 2FL with respect to anastomosis leakage and recurrent nerve palsy (Table 2). Chylothorax and pulmonary complications were not statistically significantly different between 3FL and 2FL.

Meta-analysis of studies on subgroups

There were insufficient data available for subgroup analysis; therefore, only data pertaining to recurrent laryngeal lymph node positivity/negativity were integrated for meta-analysis. Three studies were included in the positive group[10,17,18], including 107 3FL patients and 92 2FL patients. Meta-analysis of these 3 studies showed statistically significant differences between 3FL and 2FL because the OS rate in the 3FL group was superior with a pooled 1-year RR of 1.29 (95%CI: 1.08-1.53; $P = 0.004$; fixed-effects model) and statistical heterogeneity ($P = 0.80$, I2 = 0%), and a 3-year RR of 6.80 (95%CI: 2.99-15.46; $P < 0.00001$; fixed-effects model) and statistical heterogeneity ($P = 0.98$; $I^2 = 0\%$). For the negative group, 2 studies were analyzed[10,17], including 176 3FL patients and 271 2FL patients. Meta-analysis of the 2 studies showed statistically significant differences between 3FL and 2FL with a better OS rate in the 3FL group, a pooled 1-year RR of 1.14 (95%CI: 1.03-1.27; $P = 0.01$, fixed-effects model) with statistical heterogeneity ($P = 0.86$, I2 = 0%), and a 3-year RR of 1.92 (95%CI: 0.56-6.53; $P = 0.30$; random-effects model) with statistical heterogeneity ($P = 0.003$; $I^2 = 89\%$). For the other subgroups: (1) carcinoma in the upper or middle third esophagus had a survival advantage with 3FL[20,23,30]; (2) early superficial carcinoma confined to the mucosa had an equal OS rate between 3FL and 2FL[31]; and (3) poor prognostic subgroups had metastatic nodes in all 3 fields and the lower-third of

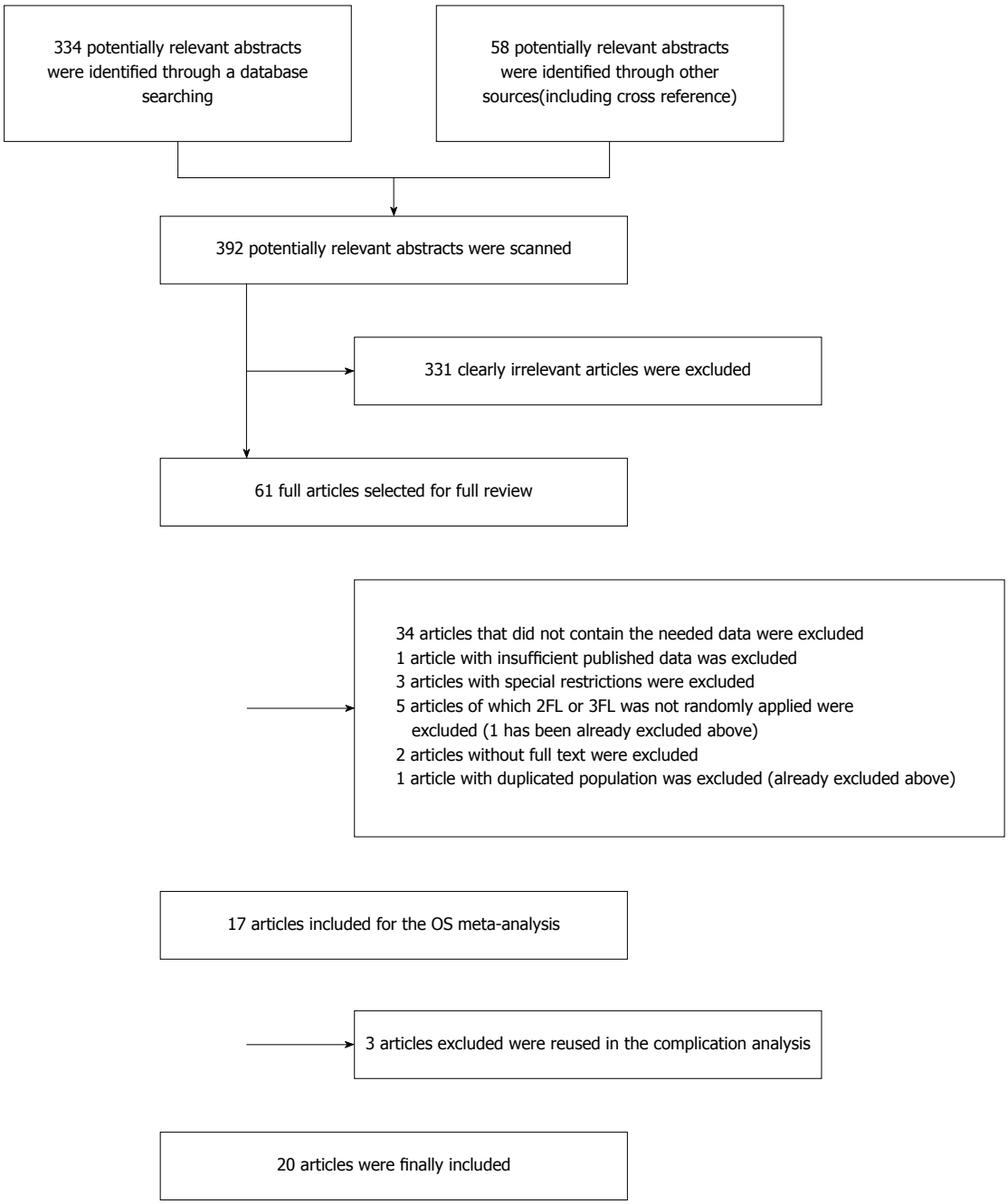

Figure 1 Flow diagram of the search strategy and study selection.

tumors had positive cervical nodes with the involvement of ⩾ 5 lymph nodes. The subgroups had equal OS rates between 3FL and 2FL[32].

DISCUSSION

Surgery for esophageal cancer includes removal of the primary lesion and lymph node dissection; however, there is a long-standing debate concerning application of 3FL, which was initiated at Chiba University (Chiba-shi, Japan) in 1983[25]. This method was based on the observation that the relapse rates at the cervical nodes were 30%-40%, which presented a significant obstacle in the improvement of surgical results[5,6]. After 1983, 3FL was widely employed in Japan, but not worldwide until recently because of lack of evidence. As mentioned above, esophagectomy is technically demanding, and few centers can recruit a sufficient number of patients to perform randomized clinical trials that can withstand scrutiny; thus, only 2 randomized trials to date have been published that compared 3FL with 2FL. One trial showed a survival advantage for 3FL; however, patients treated with 2FL were older and had more proximal tumors[8]. In the second trial, the 5-year OS rates were not statistically different between 3FL and 2FL (66.2% and 48%, respectively)[7]. These limited randomized trials were, however, insufficient to conclude that 3FL was advantageous. As there are many observational studies

Table 1 Characteristics of the studies include in the meta-analysis

Study	Year	Journal	Study design	Region	Study population, *n*	Operation time
Li *et al*[10]	2012	*J Surg Oncol*	Obser.	China	3FL: 136; 2FL: 227	2003-2010
Thakur *et al*[9]	2011	*Indian J Cancer*	Obser.	Nepal	3FL: 61; 2FL: 21	2003-2011
Shim *et al*[12]	2010	*J Thorac Oncol*	Obser.	South Korea	3FL: 57; 2FL: 34	1994-2007
Zhang *et al*[11]	2008	*Zhonghua Zhongliu Zazhi*	Obser.	China	3FL: 60; 2FL: 62	2001-2006
Igaki *et al*[13]	2004	*Ann Surg*	Obser.	Japan	3FL: 101; 2FL: 55	1988-1997
Noguchi *et al*[14]	2004	*Dis Esophagus*	Obser.	Japan	3FL: 68; 2FL: 78	1990-2001
Hagry *et al*[15]	2003	*Eur J Cardiothorac Surg*	Obser.	Belgium	3FL: 34; 2FL: 38	1975-2001
Gradauskas *et al*[16]	2002	*Medicina* (Kaunas)	Obser.	Lithuania	3FL: 23; 2FL: 19	1997-2002
Shiozaki *et al*[17]	2001	*Dis Esophaguss*	Obser.	Japan	3FL: 129; 2FL: 123	1985-1998
Tabira *et al*[18]	1999	*J Cardiovasc Surg*	Obser.	Japan	3FL: 66; 2FL: 86	1983-1996
Kawahara *et al*[19]	1998	*J Surg Oncol*	Obser.	Japan	3FL: 44; 2FL: 44	1974-1995
Nishihira *et al*[7]	1998	*Am J Surg*	RCT.	Japan	3FL: 32; 2FL: 30	1987-1994
Fujita *et al*[20]	1995	*Ann Surg*	Obser.	Japan	3FL: 63; 2FL: 65	1986-1991
Kakegawa *et al*[21]	1995	*Gan To Kagaku Ryoho*	Obser.	Japan	3FL: 124; 2FL: 107	1985-1994
Kato *et al*[22]	1995	*Ann Chir Gynaecol*	Obser.	Japan	3FL: 100; 2FL: 410	1962-1993
Akiyama *et al*[23]	1994	*Ann Surg*	Obser.	Japan	3FL: 324; 2FL: 393	1973-1993
Fujita *et al*[24]	1992	*Kurume Med J*	Obser.	Japan	3FL: 27; 2FL: 100	1982-1988
Isono *et al*[25]	1991	*Oncology*	Obser.	Japan	3FL: 1740; 2FL: 2671	1983-1989
Kato *et al*[8]	1991	*Ann Thorac Surg*	RCT	Japan	3FL: 77; 2FL: 73	1985-1989
Ando *et al*[26]	1989	*Nihon Geka Gakkai Zasshi*	Obser.	Japan	3FL: 22; 2FL: 56	1984-1988

Obser: Observational studies; 3FL: 3-field lymphadenectomy; 2FL: 2-field lymphadenectomy.

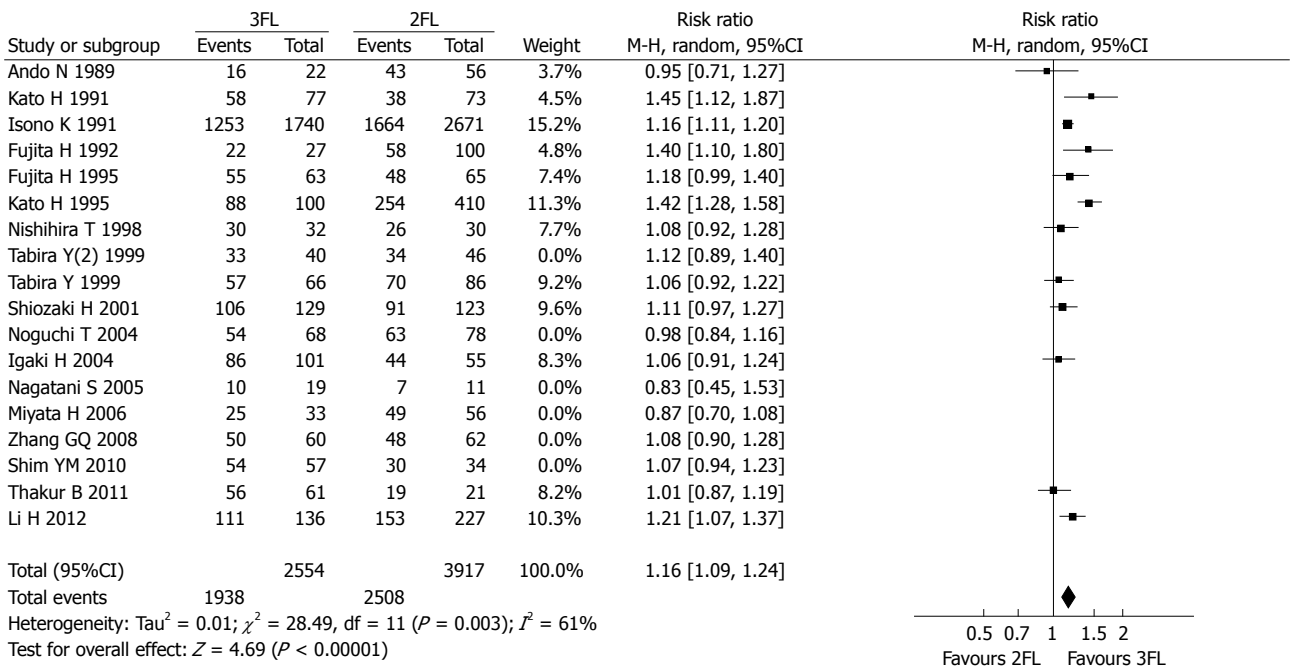

Figure 2 Forest plot of the 1-year overall survival rate. 3FL: 3-field lymphadenectomy; 2FL: 2-field lymphadenectomy.

comparing 3FL and 2FL, we performed the present meta-analysis to synthesize data to yield more comprehensive and credible results.

Meta-analysis serves as a valuable tool for studying rare and unintended treatment effects and extends prior randomized and nonrandomized studies by permitting synthesis of data and providing more stable estimates of effects. To the best of our knowledge, this is the first meta-analysis of published studies to compare 3FL and 2FL for esophageal cancer and provide evidence for the comparison of OS, postoperative complications, and subgroups.

This meta-analysis brings together all currently available data from randomized trials and observational studies comparing 2FL and 3FL in esophageal carcinoma patients, thereby providing reliable assessment of the role of 3FL. Through this meta-analysis, we discussed the 3 queries mentioned above. First, regarding the effect of 3FL on the OS rate, the present study revealed that 3FL had a significant improvement in the 1-, 3-, and 5-year OS rates (RR = 1.16, 1.44, and 1.37, respectively). However, we questioned the credibility of the 3 aspects of

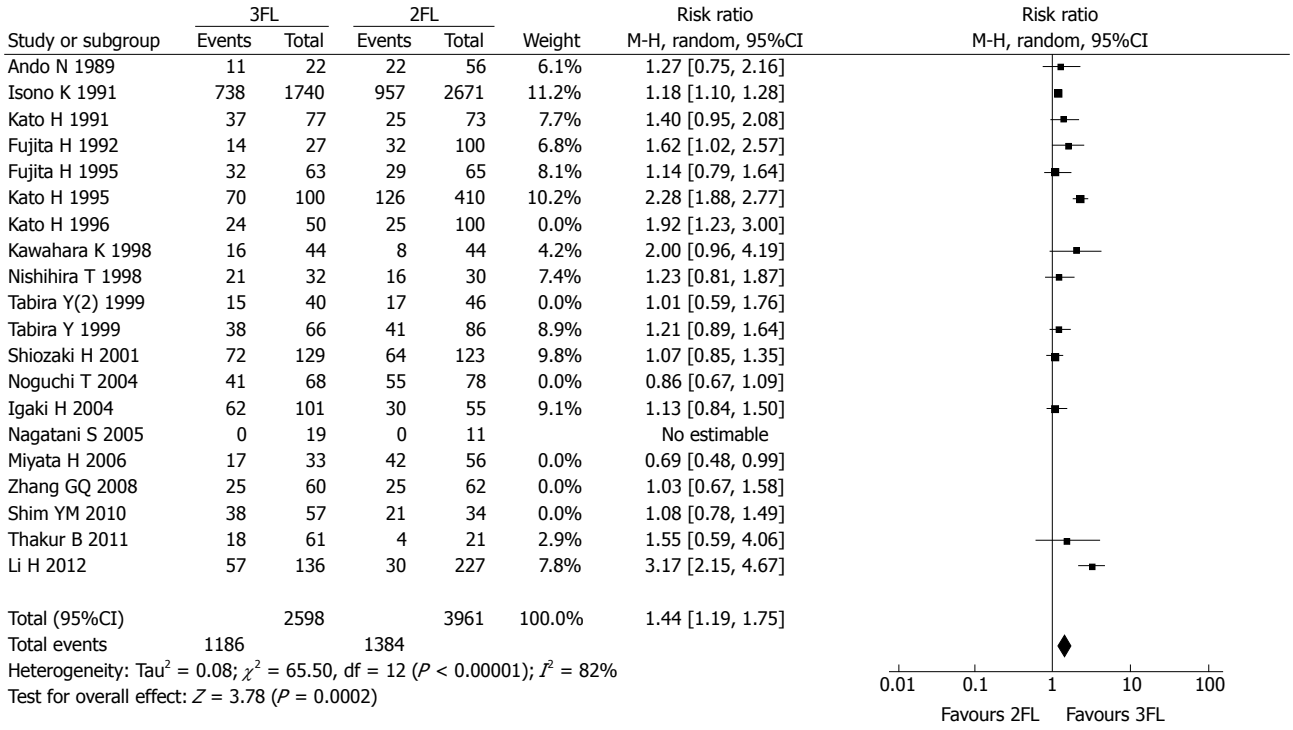

Figure 3 Forest plot of the 3-year overall survival rate. 3FL: 3-field lymphadenectomy; 2FL: 2-field lymphadenectomy.

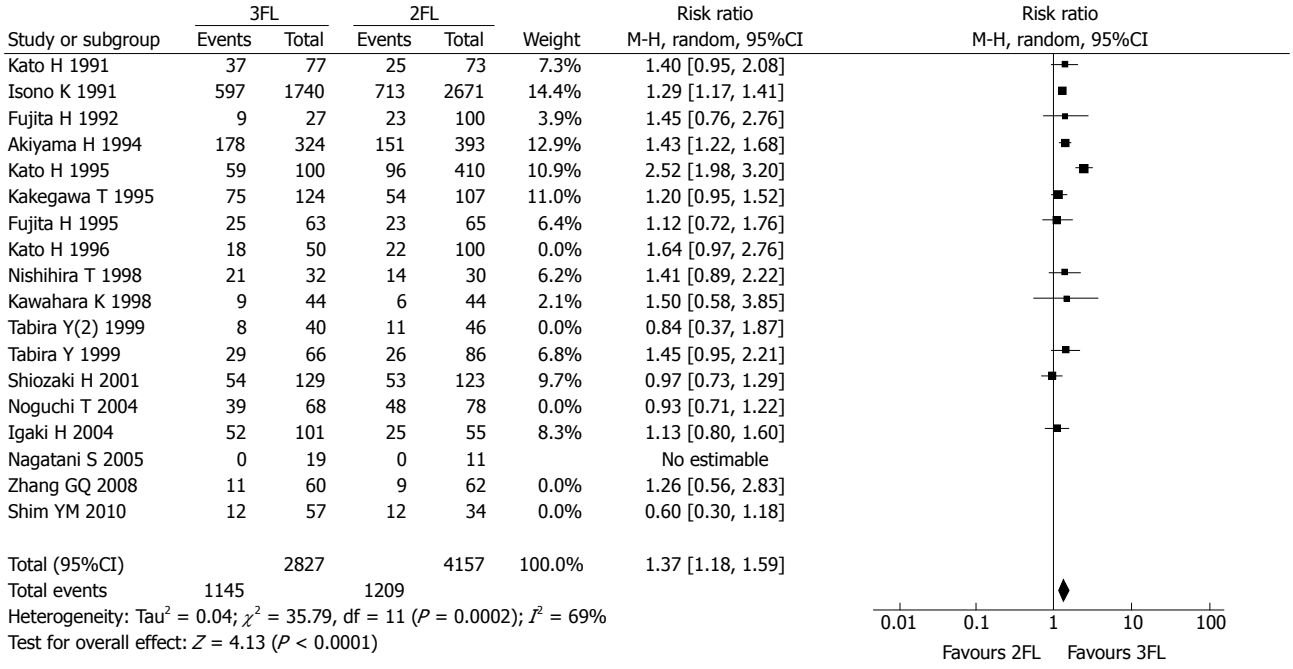

Figure 4 Forest plot of the 5-year overall survival rate. 3FL: 3-field lymphadenectomy; 2FL: 2-field lymphadenectomy.

these results. The first was the high degree heterogeneity of all the 3 outcomes. For meta-analysis of mostly observational studies, when outcome heterogeneity is particularly problematic, a single summary measure is likely inappropriate. Thus, evaluating heterogeneity becomes a key issue. Although we did our best to exclude articles that did not meet the selection criteria, the heterogeneity may be because of the following factors: (1) different types, stages, and locations of esophageal carcinoma in each observational study; (2) different patient ethnicities with different genotypes and proportion of tumor types; and (3) different institutions, surgeons with unequal skills, and different operative durations. However, the amount of information was not sufficient for stratifying or regression analysis. The second aspect was derived from theoretical justification. Although we could not perform

Table 2 Results of meta-analysis of studies for postoperative complications

Complications	Studies	Participants		Fixed-effects model		Random-effects model		Tests of homogeneity			
		3FL	2FL	RR	95%CI	RR	95%CI	*Q*	df	*P*	I^2 (%)
Recurrent nerve palsy	10	2320	3534	1.43	1.28 to 1.60	1.48	1.13-1.92	19.05	9	0.02	53
Anastomosis leak	10	608	926	1.26	1.05 to 1.52	1.32	0.97-1.81	14.53	9	0.09	38
Pulmonary complications	12	2370	3653	0.88	0.80 to 0.98	0.93	0.75-1.16	13.38	11	0.27	18
Chylothorax	6	458	699	0.77	0.32 to 1.85	0.87	0.33-2.26	3.05	5	0.69	0

3FL: 3-field lymphadenectomy; 2FL: 2-field lymphadenectomy.

3FL analysis because of frequent recurrence in the cervical lymph nodes, 2 studies on recurrence patterns after esophagectomy reported recurrence rates of 7% and 1%, which were both significantly lower than the 30% incidence rate expected from cervical metastases[33,34]. Finally, on the basis of the funnel plot results, we concluded that publication bias occurred in all the 3 outcomes.

The second part of this analysis compared postoperative complications between 2FL and 3FL. Our results determined that 3FL had more complications than 2FL in anastomosis leak and recurrent nerve palsy, while the incidences of chylothorax and pulmonary complications were not significantly different. Heterogeneity was not high because less mixed factors may have affected the result. The result was much less controversial and presents an obvious disadvantage of 3FL.

Treatment complications are often detrimental. For example, recurrent laryngeal nerve palsy due to extensive dissection of the recurrent laryngeal nerve chain remains the main concern and can affect up to 70% of cases[20]. Recurrent laryngeal nerve palsy impedes not only immediate postoperative recovery but also long-term quality of life in terms of speech, swallowing, and respiratory functions.

For the last query, we identified patients who would likely benefit from 3FL. As previously mentioned, much current research has focused on identifying optimal patients through subgroup analysis. An important study that evaluated recurrent nerve nodal involvement in 86 3FL patients identified a relationship between thoracic recurrent nerve nodal involvement and cervical metastases. Only 11% of the 63 patients without thoracic recurrent nodal involvement had positive cervical nodes, in contrast to 43% of the 23 patients with recurrent thoracic nodal disease and positive cervical nodes. A "sentinel node" concept was proposed to guide the addition of cervical lymphadenectomy[35]. However, from our results, we could not conclude that 3FL benefited only positive, but not negative, cervical nodes based on the available data. As to the other subgroups, limited data were derived from published studies; thus, they were insufficient to draw definite conclusions.

The main purpose of the present meta-analysis was to present all available evidence in a systematic, quantitative, and unbiased fashion to establish the following 3 points: the effect of 3FL on the OS rate, identification of postoperative complications of 3FL compared to 2FL, and description of patient characteristics of those who will likely benefit from 3FL. However, because of limitations of the available data, only the second query was clearly answered. For the first query, we could only determine that 3FL had better 1-, 3-, and 5-year OS rates compared to 2FL, when all currently available data were integrated. However, the credibility of the results remains controversial. Clinicians can make treatment decisions based on this evidence and consultations with patients on their perceived treatment outcomes.

COMMENTS

Background

Debate about the application of 3-field lymphadenectomy (3FL) for esophageal cancer has been heated for a long time, because the advantages and disadvantages still remain controversial when compared to the traditional 2-field lymphadenectomy (2FL).

Research frontiers

Over the decades, many observational studies comparing 3FL and 2FL have been performed to figure out whether 3FL was advantageous. Moreover, a few randomized trials were recently performed to investigate it. However, those are insufficient to reach a precise conclusion.

Innovations and breakthroughs

This is a meta-analysis of published studies to compare 3FL and 2FL for esophageal cancer and provide evidence for the comparison of overall survival, postoperative complications, and subgroups. Based on this meta-analysis, 3FL had better 1-, 3-, and 5-year overall survival rates compared to 2FL while 3FL had more complications than 2FL in anastomosis leak and recurrent nerve palsy.

Applications

3FL has better overall survival rates but more complications. Clinicians can make treatment decisions based on this evidence and consultations with patients on their acceptable treatment outcomes.

Terminology

3FL is dissecting lymph node of the bilateral cervical, mediastinal, and abdominal regions, with the theoretical justification that relapse of cervical lymph node occur frequently.

Peer review

The authors present a meta-analysis using published data on 3FL *vs* 2FL in esophageal carcinoma patients. End points of this meta-analysis were 1-, 3-, and 5-year overall survival rates and postoperative complications. This is an important clinical question and the results of this analysis will likely have an impact on clinical decisions in the future. The meta-analysis was conducted properly, objectively and the results are valid and significant. The conclusions of the manuscript are accurate, and supported by the data.

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META-ANALYSIS

Role of protective stoma in low anterior resection for rectal cancer: A meta-analysis

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Abstract

AIM: To provide a comprehensive evaluation of the role of a protective stoma in low anterior resection (LAR) for rectal cancer.

METHODS: The PubMed, EMBASE, and MEDLINE databases were searched for studies and relevant literature published between 2007 and 2014 regarding the construction of a protective stoma during LAR. A pooled risk ratio (RR) with 95% confidence intervals (CIs) was used to assess the outcomes of the studies, including the rate of postoperative anastomotic leakage and reoperations related to leakage. Funnel plots and Egger's tests were used to evaluate the publication biases of the studies. *P* values < 0.05 were considered statistically significant.

RESULTS: A total of 11 studies were included in the meta-analysis. In total, 5612 patients were examined, 2868 of whom had a protective stoma and 2744 of whom did not. The sample size of the studies varied from 34 to 1912 patients. All studies reported the number of patients who developed an anastomotic leakage and required a reoperation related to leakage. A random effects model was used to calculate the pooled RR with the corresponding 95%CI because obvious heterogeneity was observed among the 11 studies (I^2 = 77%). The results indicated that the creation of a protective stoma during LAR significantly reduces the rate of anastomotic leakage and the number of reoperations related to leakage, with pooled RRs of 0.38 (95%CI: 0.30-0.48, *P* < 0.00001) and 0.37 (95%CI: 0.29-0.48, *P* < 0.00001), respectively. The shape of the funnel plot did not reveal any evidence of obvious asymmetry.

CONCLUSION: The presence of a protective stoma effectively decreased the incidences of anastomotic leakage and reoperation and is recommended in patients undergoing low rectal anterior resections for rectal cancer.

Key words: Protective stoma; Low anterior resection; Rectal cancer; Complication; Meta-analysis

Core tip: The use of a protective stoma is an effective application that can reduce the rate of anastomotic leakage in patients who receive low anterior resection for rectal cancer. The morbidity associated with protective stomas and the complications of stoma closure are negligible compared with the reoperations required for anastomosis leakage in the absence of a protective stoma. Therefore, the presence of a non-functioning stoma can be useful for patients undergoing rectal surgery and is recommended during low anterior resections for rectal cancer.

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INTRODUCTION

Low anterior resection (LAR) is the standard operation for rectal cancer and allows an anastomosis to be created at a lower level, thereby preserving the anal sphincter[1]. Nonetheless, anastomotic leakage remains one the most significant complications after LAR. Anastomotic leakage is defined as a communication between the intra- and extraluminal compartments owing to a defect in the integrity of the intestinal wall at the anastomosis between the colon and rectum or the colon and anus[2]. In the last decade, the problem of anastomotic leakage has been widely addressed in multiple symposia and many publications[3]. Leakage rates from 3% to > 20% leading to substantial postoperative morbidity and mortality have been reported[4,5]. Even experienced surgeons sometimes find it difficult to predict which patients will develop an anastomotic leak, and such leaks may occur even when the anastomosis is technically sound or when the risk factors for leakage are absent. Studies have demonstrated that such low anastomoses carry a considerably higher risk of anastomotic leakage[6]. Leakage can increase morbidity and mortality, prolong the duration of the hospital stay, and affect short- or long-term quality of life[7,8]. There is also evidence for an increased risk of local cancer recurrence and decreased long-term survival after leakage[9-11].

Many solutions have been sought to prevent or diminish anastomotic leakage, such as mechanical bowel preparation, drains, and intra-luminal devices. Some surgeons use a protective stoma after LAR to prevent anastomotic leakage in the hope that by diverting the fecal stream and keeping the anastomosis free of material, leakage will be less likely. While other surgeons have reported that covering the protective stoma had no influence on anastomotic leakage and reoperation rates, the further complications that can be caused by the stoma itself should not be ignored, as they include discomfort and inconvenience, high output with consequent dehydration, and anastomotic complications at the stoma closure site[12-18].

Although protective stomas are widely used in LAR for rectal cancer, it remains unclear whether such protective stomas are useful for patients. Therefore, we performed this meta-analysis to investigate whether a protective stoma affects the outcomes of patients undergoing LAR for rectal cancer.

MATERIALS AND METHODS

Publication search

The PubMed, EMBASE, and MEDLINE databases and the Cochrane Central Register of Controlled Trials were searched to locate articles (published between January 2007 and January 2014), including articles referenced in the publications. The search strategy included the following keywords in various combinations: "low anterior resection", "stoma", "protective stoma", "rectal cancer", and "anastomotic leakage". Internet search engines were also used to perform a manual search for abstracts from international meetings, which were then downloaded and studied.

Inclusion and exclusion criteria

The inclusion criteria were as follows: studies that compared LAR with or without a protective stoma and recent clinical trials from 2007 to 2014. When a study reporting the same patient cohort was included in several publications, only the most recent or most complete study was selected. The exclusion criteria were as follows: studies of case reports, letters, or reviews without original data; non-English papers; animal or laboratory studies; non-rectal cancer proctectomy; and articles that were not full-text or non-comparative studies. If any doubt of suitability remained after the abstract was examined, the full manuscript was obtained[19].

Data extraction

Two review authors assessed the methodological quality of the potentially eligible studies without considering the results. The extracted data were then crosschecked between the two authors to rule out any discrepancies. Data were extracted independently from each of the included studies regarding the following: first authors' surname, publication year, sample size, number of patients who developed an anastomotic leak and required a reoperation related to leakage after LAR, and whether a protective stoma was involved. Disagreements were discussed by the authors and resolved by consensus.

Statistical analysis

Statistical analysis was performed using the Review Manager (RevMan) software, version 5.0 (The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). A pooled risk ratio (RR) with 95% confidence intervals (CIs) was used to assess the outcomes of the studies. I^2 statistics were used to evaluate the between-study heterogeneity analysis in this meta-analysis[20]. The random effects model was used when obvious heterogeneity was observed among the included studies ($I^2 > 50\%$). The fixed effects model was used when there was no significant heterogeneity between the included studies ($I^2 \leqslant 50\%$). Publication bias was estimated using a funnel plot with an Egger's linear regression test; funnel plot asymmetry on the natural logarithm scale of the RR was measured using a linear regression approach.

RESULTS

Study characteristics

The initial search retrieved a total of 164 references, and after screening the titles and abstracts of the identified

Table 1 Main characteristics of the five included studies

Ref.	Year	No. of patients	*n*		Type of operation	Leakage		Reoperation	
			Stoma	No stoma		Stoma	No stoma	Stoma	No stoma
Beirens *et al*[21]	2012	1912	1183	729	LAR	51	74	40	69
Chude *et al*[22]	2008	256	136	120	LAR	3	12	0	2
Gong *et al*[23]	2012	62	36	26	uLAR	0	5	0	2
Karahasanoglu *et al*[24]	2011	77	23	54	LAR	0	3	-	-
Lefebure *et al*[25]	2008	132	42	90	LAR	3	10	1	5
Ma *et al*[26]	2013	56	30	26	LAR	2	7	0	5
Matthiessen *et al*[27]	2007	234	116	118	LAR	12	33	12	32
Nurkin *et al*[28]	2013	1791	958	833	LAR	17	26	37	63
Seo *et al*[29]	2013	836	246	590	uLAR	1	22	-	-
Shiomi *et al*[30]	2010	222	80	142	LAR	3	17	0	14
Ulrich *et al*[31]	2009	34	18	16	LAR	1	6	0	6

LAR: Low anterior resection; uLAR: Ulter-low anterior resection.

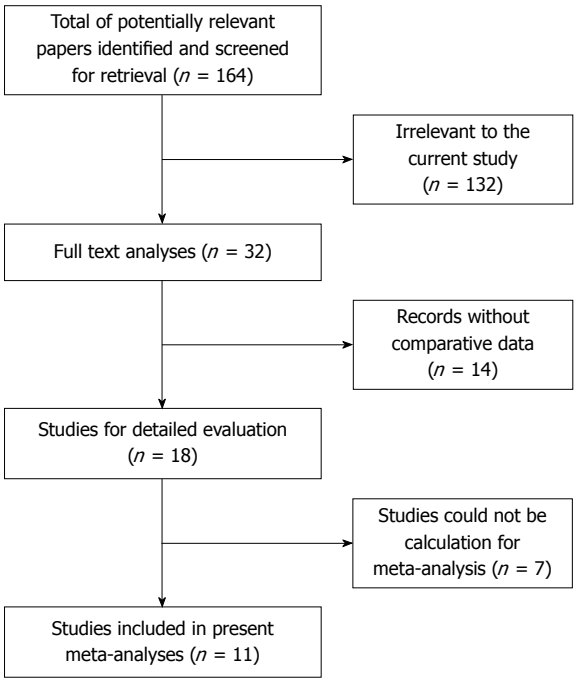


Figure 1 Flow chart of study selection.

articles, 132 studies were excluded because they were not related to the current study. Of these studies, 56 were reviews, 35 were case reports, 11 were animal studies, and 20 included cases of non-rectal cancer proctectomy. Upon further review, 14 additional studies were excluded because they did not include comparative data. We evaluated 18 potential candidate studies in the full text, 7 of which were not published in English. Finally, 11 studies[21-31] were included in this meta-analysis, all of which were published between 2007 and 2014. The flow chart of study selection is presented in Figure 1.

There were three RCTs and eight non-randomized studies involving a total population of 5612 patients, among whom 2868 had a protective stoma and 2744 did not. The sample sizes of the studies varied from 34 to 1912 patients. All studies reported the number of patients who developed an anastomotic leak and required a reoperation after LAR. Moreover, some studies reported the risk factors for anastomotic leakage and short-term mortality following LAR, although these data were not compared in this meta-analysis. The main characteristics of the included studies are summarized in Table 1.

Meta-analysis results

All of the studies reported results on clinical anastomotic leakage and reoperation. The random effects model was used to calculate the pooled RR with the corresponding 95%CI because obvious heterogeneity was observed among those 11 studies (I^2 = 77%). The results indicated that the absence of a protective stoma was associated with a higher incidence of anastomotic leakage and reoperation, with pooled RRs of 0.38 (95%CI: 0.30-0.48, $P < 0.00001$, Figure 2A) and 0.37 (95%CI: 0.29-0.48, $P < 0.00001$, Figure 2B), respectively. The present meta-analysis revealed that a statistically significant advantage was conferred by a protective stoma in patients undergoing LAR.

Publication bias

Funnel plots and Egger's tests were used to evaluate the publication bias within the literature. The shape of the funnel plot did not reveal any evidence of obvious asymmetry (Figure 3).

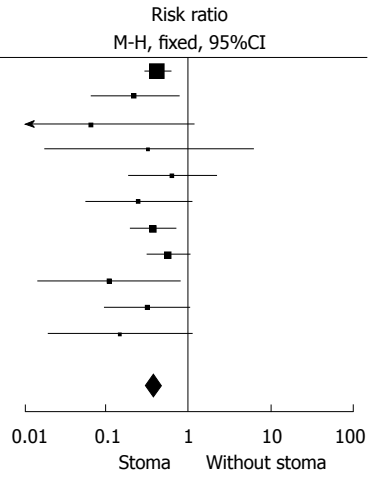
DISCUSSION

Total mesorectal excision (TME) in combination with LAR plays an important role in the treatment of patients with rectal cancer[32]. Considering the incidence of rectal cancer, the improvements in medical instruments, and the higher requirements of patients regarding quality of their post-surgical lives, ultralow anterior rectal resection has become the primary low sphincter-preserving procedure. However, this procedure can also increase the risk of anastomotic leakage[33]. Possible factors contributing to an increased leakage rate include the reduced blood supply of the anorectal remnant and the large pelvic space after TME, which may predispose a patient to fluid accumulation and pelvic infection[34]. Symptomatic anastomotic leakage is the most feared complication and

A

Study or subgroup	Stoma Events	Stoma Total	Without stoma Events	Without stoma Total	Weight	Risk ratio M-H, fixed, 95%CI
Beirens 2011	51	1183	74	729	41.9%	0.42 (0.30, 0.60)
Chude 2008	3	136	12	120	5.8%	0.22 (0.06, 0.76)
Gong 2012	0	36	5	26	2.9%	0.07 (0.00, 1.15)
Karahasanoglu 2011	0	23	3	54	1.0%	0.33 (0.02, 6.10)
Lefebure 2008	3	42	10	90	2.9%	0.64 (0.19, 2.22)
Ma 2013	2	30	7	26	3.4%	0.25 (0.06, 1.09)
Matthiessen 2007	12	116	33	118	15.0%	0.37 (0.20, 0.68)
Nurlin 2013	17	958	26	833	12.7%	0.57 (0.31, 1.04)
Seok 2013	1	246	22	590	5.9%	0.11 (0.01, 0.80)
Shiomi 2010	3	80	17	142	5.6%	0.31 (0.09, 1.04)
Ulrich 2009	1	18	6	16	2.9%	0.15 (0.02, 1.10)
Total (95%CI)		2868		2744	100.0%	0.38 (0.30, 0.48)
Total events	93		215			

Heterogeneity: χ^2 = 7.78, df = 10 (P = 0.65); I^2 = 0%
Test for overall effect: Z = 7.89 (P < 0.00001)



B

Study or subgroup	Stoma Events	Stoma Total	Without stoma Events	Without stoma Total	Weight	Risk ratio M-H, fixed, 95%CI
Beirens 2011	40	1183	69	729	39.4%	0.36 (0.24, 0.52)
Chude 2008	0	136	2	120	1.2%	0.18 (0.01, 3.64)
Gong 2012	0	36	2	26	1.3%	0.15 (0.01, 2.92)
Lefebure 2008	1	42	5	90	1.5%	0.43 (0.05, 3.55)
Ma 2013	0	30	5	26	2.7%	0.08 (0.00, 1.37)
Matthiessen 2007	12	116	32	118	14.7%	0.38 (0.21, 0.70)
Nurlin 2013	37	958	63	833	31.1%	0.51 (0.34, 0.76)
Shiomi 2010	0	80	14	142	4.8%	0.06 (0.00, 1.01)
Ulrich 2009	0	18	6	16	3.2%	0.07 (0.00, 1.13)
Total (95%CI)		2599		2100	100.0%	0.37 (0.29, 0.48)
Total events	90		198			

Heterogeneity: χ^2 = 7.24, df = 8 (P = 0.51); I^2 = 0%
Test for overall effect: Z = 7.99 (P < 0.00001)

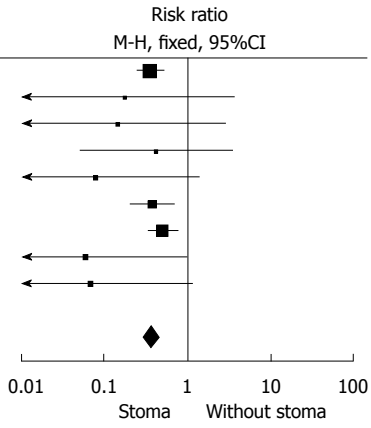


Figure 2 Forest plot. A: Forest plot for a comparison of the study outcomes of low anterior resection with or without stoma *vs* anastomotic leakage; B: Forest plot of the study outcomes of low anterior resection with or without stoma *vs* reoperation rate. Risk ratios are shown with 95%CIs.

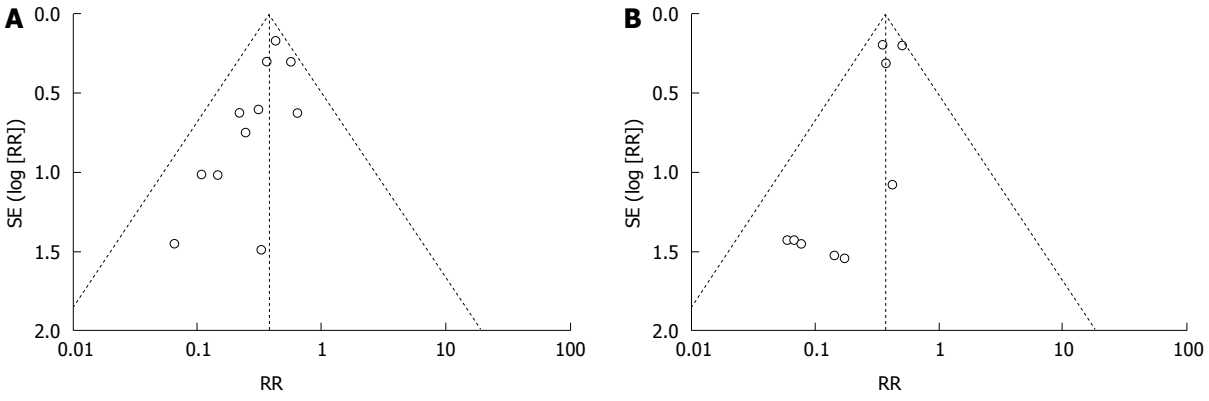


Figure 3 Funnel plot. A: Funnel plot for the publication bias test of outcomes of low anterior resection with or without stoma *vs* anastomotic leakage; B: Funnel plot for the publication bias test regarding outcomes of low anterior resection with or without stoma *vs* reoperation rate. RR: Risk ratio.

has been reported to occur in 1% to 24% of patients[35]; when present, the associated risk of postoperative mortality is increased to 6% to 22%[36].

The use of a non-functioning stoma in LAR has been considered to decrease the leakage rate and its fatal consequences by keeping the distal anastomosis relatively "clean" and reducing the intraluminal pressure of the bowel[33,37]. Moreover, a protective stoma can mitigate its inherent consequences[38]. Nonetheless, the value of a protective stoma has been the subject of controversy for many years. Some surgeons do not choose fecal diversion because fecal diversion requires the patient to undergo two surgeries and because a protective stoma does not reduce the leakage rate after LAR. Previous publications have reported that the overall leakage and reoperation rates were similar in patients with or without a protective

stoma[39]. In addition, ostomy construction and closure is associated with considerable morbidity and increased costs[40]. The potential disadvantages of a protective stoma include the need for another operation, a longer hospital stay, and ostomy-related complications, such as prolapse, retraction, necrosis, stenosis, peristomal abscess, parastomal hernia, and skin problems. Therefore, the benefits of a protective stoma in decreasing the rate of anastomotic leakage must be balanced against the morbidity of its construction and closure. Furthermore, even when a non-functioning stoma is constructed, there remains a considerable risk of anastomotic leakage[41]. Thus, the benefits conferred by a protective stoma have not been unequivocally demonstrated. To further evaluate this argument, we completed the present meta-analysis including all of the relevant data available. The straightforward conclusion of the 11 included studies is that the creation of a protective stoma in LAR significantly reduces the rate of anastomotic leakage and the number of reoperations related to leakage.

Overall, this meta-analysis has certain limitations. First, there was only a small number of RCTs available for inclusion. Although no detectable publication bias was observed in the funnel plot, the overall methodological quality and reporting of the included studies were poor. Because of limitations of medical ethics, not all of the studies were randomized controlled trials, and the sample size of some studies was rather low[42]. Second, among the studies included in the analysis, some included only patients undergoing elective surgery, whereas others included patients undergoing elective or emergency surgery for colorectal anastomoses. This discrepancy may have introduced some bias that would have contributed significantly to the analysis. Third, considerable selection bias existed in some of the included studies. Surgeons relied on their personal experiences to predict the patients who were at high risk of an anastomotic leakage, which may have been inaccurate and is suggestive of a potential selection bias among those who underwent stoma formation. Anastomotic leakage is unpredictable, as it can also occur in patients with no obvious risk factors. However, in some of the retrospective and prospective studies, the so-called high-risk patients were included in the protective stoma group.

In the light of these findings, the use of a protective stoma is an effective approach for reducing the rate of anastomotic leakage in patients who undergo LAR for rectal cancer. The morbidity associated with protective stoma and the complications of stoma closure are negligible compared with the reoperations required for anastomosis leakage in the absence of protective stoma. Therefore, non-functioning stoma can be useful for patients undergoing rectal surgery and is recommended during a LAR for rectal cancer. Future randomized controlled trials are needed to address the long-term mortality and quality of life issues related to protective stoma in LAR for rectal cancer.

COMMENTS

Background

Anastomotic leakage remains one the most significant complications after low anterior resection (LAR). Recent studies have demonstrated that the use of a protective stoma can reduce morbidity in LAR for rectal cancer, but the necessity of this procedure remains controversial.

Research frontiers

Over the last decade, the problem of anastomotic leakage has been widely addressed in multiple symposia and many publications. Although the use of a protective stoma is widely applied in LAR for rectal cancer, it remains unclear whether the protective stoma is useful for patients. Therefore, we performed this meta-analysis to investigate whether a protective stoma affects the outcomes of patients undergoing LAR for rectal cancer.

Innovations and breakthroughs

Based on this meta-analysis, the use of a protective stoma is an effective means of reducing the rate of anastomotic leakage in patients who receive LAR for rectal cancer. The morbidity associated with protective stoma usage and the complications of stoma closure are negligible compared with the reoperations required for anastomosis leakage in the absence of a protective stoma.

Applications

A protective stoma can be useful for patients undergoing rectal surgery and is recommended during LAR for rectal cancer. Future randomized controlled trials are needed to address the long-term mortality and quality of life issues related to protective stoma usage in LARs for rectal cancer.

Peer review

This is a meta-analysis study on the necessity of protective stoma in low anterior resection with total mesorectal excision (TME) for rectal cancer.Its publication seems important in a time of intense and controversial discussion about the necessity of protective stoma in low anterior resection with TME for rectal cancer.

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CASE REPORT

Eosinophilic esophagitis in patients with esophageal atresia and chronic dysphagia

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Abstract

Esophageal atresia (EA) is defined as a discontinuity of the lumen of the esophagus repaired soon after birth. Dysphagia is a common symptom in these patients, usually related to stricture, dysmotility or peptic esophagitis. We present 4 cases of patients with EA who complained of dysphagia and the diagnosis of Eosinophilic esophagitis (EoE) was made, ages ranging from 9 to 16 years. Although our patients were on acid suppression years after their EA repair, they presented with acute worsening of dysphagia. Esophogastroduodenoscopy and/or barium swallow did not show stricture and biopsies revealed elevated eosinophil counts consistent with EoE. Two of 4 patients improved symptomatically with the topical steroids. It is important to note that all our patients have asthma and 3 out of 4 have tested positive for food allergies. One of our patients developed recurrent anastomotic strictures that improved with the treatment of the EoE. A previous case report linked the recurrence of esophageal strictures in patients with EA repair with EoE. Once the EoE was treated the strictures resolved. On the other hand, based on our observation, EoE could be present in patients without recurrent anastomotic strictures. There appears to be a spectrum in the disease process. We are suggesting that EoE is a frequent concomitant problem in patients with history of congenital esophageal deformities, and for this reason any of these patients with refractory reflux symptoms or dysphagia (with or without anastomotic stricture) may benefit from an endoscopic evaluation with biopsies to rule out EoE.

Key words: Eosinophilic esophagitis; Esophageal atresia; Tracheoesophageal fistula; Dysphagia

Core tip: Dysphagia is frequently seen in patients with repaired esophageal atresia (EA). It has been attributed to recurrent strictures, poor esophageal motility and persistent gastroesophageal reflux disease. Anastomitic strictures are common after repair of a gap that is greater than 2.5 cm contributing to this complication. The pathophysiology of later onset dysphagia is not well defined. Eosinophilic esophagitis (EoE) has been reported to play a role in the reoccurrence of strictures in patients with EA. It is very likely that if these patients are treated for EoE early in the course of the disease, stricture formation might be prevented.

Kassabian S, Baez-Socorro V, Sferra T, Garcia R. Eosinophilic esophagitis in patients with esophageal atresia and chronic dysphagia. *World J Gastroenterol* 2014; 20(47): 18038-18043 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/18038.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.18038

INTRODUCTION

Esophageal atresia (EA) and associated tracheoesopha-

geal fistula (TEF) is a frequent congenital malformation repaired soon after birth[1-19]. Dysphagia occurs commonly in infants and children with a history of EA repair. In the early postoperative period, this symptom is most frequently related to an anastomotic stricture. Later in life, dysmotility and peptic esophagitis have been found to contribute to the development of dysphagia[3,20-22]. Eosinophilic esophagitis (EoE) is a clinicopathological diagnosis, including dysphagia as a common symptom along with abnormal endoscopic findings and esophageal eosinophilia. Its presentation in patients with previous EA has not been fully recognized. We describe 5 patients between the ages of 1-16 years, with a history of EA who presented with dysphagia, not associated with anastomotic strictures. Each patient was subsequently diagnosed with EoE. Our objective is the highlight the possible association of these two disorders and to emphasize the importance of endoscopic evaluation with biopsies in patients with a history of EA who present with dysphagia.

CASE REPORT

Case 1

A 10-year-old female with VATER association presented with 1 year history of dysphagia, and recent onset of postprandial chest pain. She was born at 29 wk estimated gestational age. She underwent EA and TEF repair soon after birth. During infancy, she experienced recurrent TEFs requiring repair. She developed significant gastroesophageal reflux that was felt to contribute to frequent episodes of aspiration pneumonia and underwent an anti-reflux surgical procedure. She had poorly controlled asthma, eczema, and a history of an elevated serum IgE (2332 IU/mL). The treatment for her asthma included previous use of steroids for asthma exacerbations as well as daily medication which included cetirizine, inhaled fluticasone/salmeterol, montelukast, and albuterol whenever needed. At the time of her presentation her complete blood count and differential were normal. A barium esophagram demonstrated incomplete clearance of the contrast without evidence of an esophageal stricture. Esophagogastroduodenoscopy (EGD) performed after a two month course of lansoprazole, revealed erythema with no thickened folds or erosions. The anti-reflux surgical procedure did not appear to be intact and a moderate hiatal hernia was seen. Histologically, the distal esophagus demonstrated greater than 60 eosinophils per high-power field (eos/hpf) with superficial eosinophilic infiltrate and the proximal esophagus with a maximum of 30 eos/hpf with transmural eosinophilic infiltrate. An esophageal pH impedance study conducted while on proton pump inhibitor (PPI), showed no evidence of pathologic reflux. She was diagnosed with EoE. Skin prick testing revealed reactions to beef, egg, peas and milk. These foods were eliminated from her diet. She was placed on budesonide (0.5 mg in sucralose, twice daily for one month followed by once daily for one month prior to discontinuation) in addition to a PPI. She continued to have similar symptoms with very mild relief. A follow-up examination 6 mo after her previous EGD, revealed a normal caliber esophagus with furrowing distal to the TEF repair, with normal esophageal anastomosis. Mucosal biopsies contained a maximum of 50 eos/hpf with focally superficial eosinophilic infiltrate. She was prescribed fluticasone 0.5 mg twice daily and a more restricted diet (additionally eliminating wheat, seafood, shellfish and peanuts). Nine months after the second EGD, she continued to experience the same symptoms and was unable to comply with the diet restrictions or a trial of an elemental diet. The examined esophagus appeared normal. The site of TEF repair was patent in the proximal esophagus. Biopsies contained a maximum of 42 eos/hpf with focal eosinophilic infiltrate in the surface. At this point she was referred to pediatric surgery for repair of her Nissen fundoplication, hiatal hernia and placement of a gastrostomy. After these surgical interventions she was started on an elemental formula through her gastrostomy and asked to hold oral intake with a planned EGD in the future.

Case 2

A 9-year-old male with a history of isolated EA, repaired after birth, was referred to pediatric gastroenterology for evaluation of coffee ground emesis and melena. At the time of referral he had a chronic complaint of dysphagia. He had a history of recurrent symptomatic esophageal stricture requiring dilation. The last dilation was performed 3 years prior. He had asthma, which was treated with montelukast, cetirizine, and inhaled fluticasone. He was receiving lansoprazole (15 mg, daily). This dose was increased to twice a day at the time of presentation (two weeks prior to the first EGD). He had a normal complete blood count and differential. A fluoroscopic barium swallow revealed absent esophageal peristalsis with delayed clearance of contrast from the esophagus. The esophagus was of normal caliber without stricture. At EGD, the proximal esophagus was dilated, the anastomosis site was visualized, no ulcers were noted, the distal esophageal mucosa appeared erythematous with loss of a normal vascular pattern. The biopsies did not specify the number of eos/hpf, but was read as florid intraepithelial eosinophil infiltration and superficial collections of eosinophils most consistent with EoE. He was treated with swallowed fluticasone 0.5 mg twice daily and continued acid suppression (lansoprazole 15 mg, twice daily). Radioallergosorbent test (RAST) evaluation revealed a low positive result for wheat and egg white. He was placed on a diet restricted for milk, wheat, and egg. After three months of therapy, a second EGD revealed a normal appearance to the esophageal mucosa with normal histology. Wheat was reintroduced into his diet. Two months later, a third EGD showed a ringed and furrowed esophagus. Biopsies contained epithelial hyperplasia, edema, and a dense intraepithelial eosinophil infiltrate, greater than 50/hpf. Total IgE at this time was 343, 6 food elimination was initiated, with restriction of milk, soy, egg, wheat, peanuts/tree nuts and fish/shellfish.

Case 3

A 16-year-old male with EA and TEF repaired after birth presented with dysphagia, epigastric abdominal pain, chest pressure and vomiting associated with exercise. He had a history of gastroesophageal reflux disease treated with PPI, which was discontinued at time of presentation and asthma diagnosed at 2 years of age. He required multiple esophageal dilations with the most recent one at 14 years of age. Prior to the age of 6 he experienced intermittent episodes of choking and food impaction requiring dilatation. His current therapy for asthma includes budesonide, loratadine and montelukast. Previous RAST revealed an allergy to milk and egg, but diet continued to contain these two foods. A fluoroscopic barium swallow showed an absence of esophageal motility and no focal esophageal narrowing. He was started on omeprazole 40 mg once daily for one week prior to upper endoscopy. Examination of the esophagus showed diffuse mucosal edema and linear esophageal furrowing throughout the entire length of the esophagus. There was circumferential area in the distal esophagus just above the LES which was erythematous and had mucosal erosions.

Esophageal biopsies of the proximal and distal esophagus revealed 35 eos/hpf. He was then started on fluticasone 440 mcg twice a day with complete resolution of his symptoms after 1 wk of treatment. Skin prick testing was negative. Recommendation of eliminating milk, soy, wheat, egg, peanuts/tree nuts and fish/shellfish was made, but the patient refused to follow the elimination diet. He was also continued on another 4 wk of fluticasone 440 mcg and omeprazole 40 mg once daily. Upon two month follow up he was asymptomatic, continuing his omeprazole. A follow up endoscopy was refused by the patient's parents since he was doing so well.

Case 4

A 17-year-old female with EA and TE fistula repaired after birth with a history of reflux and esophageal stricture requiring multiple dilatations. Significant past medical history of failure to thrive, asthma and multiple food allergies including milk, egg, peanut and shellfish, diagnosed by skin prick testing.

She presented with dysphagia, regurgitation and the feeling of meat getting stuck regardless of chewing very well. Her parent reported eosinophils in the esophagus in the past, with no further details. During the initial visit she was taking omeprazole 20 mg twice a day and cetirizine.

At age 13, at the time of EGD an 8.6 mm outer diameter endoscope passed easily through the anastomosis, but there was some post-endoscopic trauma. No dilatation was done during this procedure. Biopsies showed squamous hyperplasia with a moderate number of eosinophils in the proximal esophagus and chronic inflammation with patchy eosinophils in the distal esophagus; interpreted at the time as possible reflux esophagitis. Patient was changed to lansoprazole 30 mg twice daily. Patient had improvement of the reflux symptoms but the dysphagia continued.

Six months later she presented with worsening of the dysphagia requiring pneumatic dilation and triamcinolone injection in the anastomosis area. She was continued on lansoprazole and sucralfate for one week. Due to persistent symptoms of dysphagia this patient underwent several EGDs requiring dilation and triamcinolone injections every 6-8 mo over a 3-year period. The proximal esophagus began to appear furrowed, four years after initial presentation, and the biopsies showed 30 eos/hpf. At this point she was started on fluticasone, oral steroids, and diet restriction for milk, egg, peanut/tree nut and fish/shellfish, the patient had significant improvement in symptoms and complete resolution of the esophageal eosinophilia. Her last EGD showed biopsies consistent with Barrett's epithelium with no eosinophils and she has not required any further dilations in the last 15 mo.

Case 5

A 17-mo-old former 28 wk old premature male born with TEF and EA repaired soon after birth. His past medical history is significant for small ASD of no hemodynamic significance, tracheobronchomalacia, chronic lung disease of prematurity, oropharyngeal incoordination, GERD and aspiration requiring G-tube feeds.

At 8 mo of age he required hospitalization for an ALTE. During that hospitalization a pH impedance showed severe acid reflux. He was started on lansoprazole 15mg once daily. His barium swallow showed mild narrowing at the anastomosis site. His EGD showed narrowing at the anastomosis site which did not interfere with the advancement of an endoscope with an outer diameter 5.9 mm.

At 16 mo of age he presented to pediatric gastroenterology with dysphagia for solids. An upper endoscopy was scheduled and dilatation was conducted with a pneumatic dilator, biopsies were also taken during the procedure. His symptoms did not improve post-dilatation. Histology showed epithelial hyperplasia, edema, a dense intraepithelial lymphocytic infiltrate and intraepithelial eosinophils up to 20/hpf, consistent with EoE. A skin prick test was nonreactive. He was placed on an empiric diet restriction of dairy, egg and peanuts/tree nuts. Once the EoE was diagnosed, diet modifications were made, the patient's symptoms resolved within one month. Esophageal examination four months after initiation of the diet restrictions, showed a benign-appearing, intrinsic mild stenosis measuring less than one cm in length and this was traversed. The biopsies from the proximal and distal esophagus had mild basal cell hyperplasia, scattered mononuclear cells and rare eosinophils (2 eos/hpf). Egg was reintroduced into the diet and milk and dairy were still restricted, he was also continued on the lansoprazole. Three months later a repeat upper endoscopy revealed a normal appearing esophagus. Histology showed mild basal cell hyperplasia, increased mononuclear cells and rare eosinophils (2 eos/hpf).

DISCUSSION

Dysphagia is a frequent symptom in patients with repaired EA[14]. Historically this symptom has been attributed to recurrent strictures, poor esophageal motility and persistent GERD. Anastomotic strictures are frequent early complications, that present with dysphagia, in patients with EA repair, with a mean presentation age of 5 months and a frequency of 37%-57%[4,23,24], Strictures early in the life of these patients respond well to dilatations[25]. An important factor for the development of subsequent stricture is anastomotic tension which is highly correlated with gap length[4,23,24].

Gastroesophageal reflux disease (GERD) is common in this population, occurring in up to 58% of children[5]. Risk factors for GERD in these patients include low birth weight, delayed anastomosis and possibly gastrostomy tube placements[6,26]. GERD has been related as a factor for the formation of postoperative stricture and its recurrence[4,7,8,22-24,27,28]. The standardized use of PPIs appears to have decreased the prevalence of GERD related stricture formation in patients with EA[4,23,24]. Proton pump inhibitors have now become a standard treatment in all patients with EA repair[4,23,24].

It is clear that anastomotic strictures are common after repair of a gap that is greater than 2.5 cm and certain types of EA/TEF as well as vascular compromise contributing to this complication[24]. The pathophysiology of later onset of dysphagia in these patients is not well defined. Multiple publications have noted that numerous factor including dysmotility, GERD and strictures play a role[9,29,30]. We propose an additional etiology to consider when a patient complains of dysphagia. Eosinophilic esophagitis should be considered and further investigated by an upper endoscopic evaluation with proximal and distal biopsies.

In the 2011 Consensus Recommendations of the International Gastrointestinal Eosinophil Researchers defined eosinophilic esophagitis as a chronic, immune/antigen mediated, esophageal disease characterized by symptoms related to esophageal dysfunction and histologically by eosinophil-predominant inflammation[10,31]. Symptoms of EoE can be confused with GERD-like symptoms that do not respond to conventional anti-reflux therapies[1,20]. There is an increasing prevalence of EoE in recent years as well as a male predominance shown in several studies[3,11,20-22,32,33].

EoE, has been reported to play a role in the reoccurrence of strictures in patients with EA. To our knowledge there has only been a couple of case series relating EA and EoE[12-14]. Taking into account previous case series and our experience from case 4, strictures that develop later in the course of patients with EA repair and EoE do not respond as well to pneumatic dilators unless the eosinophilia is treated. It is very likely that if these patients are treated for EoE early in the course of the development of the disease, stricture formation might be prevented, although to date we do not have evidence of this.

All our patients presented with either dysphagia and/or food impaction years after initial repair. These patients had endoscopic and histological findings consistent with EoE. We did not see any evidence of significant anastomotic stricture on the esophagrams and endoscopic evaluations of our five patients. In one patient we found a narrowing of the anastomosis site that did not impede the passage of the 8.6 mm endoscope and dilation did not resolve the patient's dysphagia. Symptoms of dysphagia and reflux improved with the treatment of the EoE. Four had significant atopic history; all carrying the diagnosis of asthma and 3 out of 5 with known food allergy. Three of our 5 patients did not have stricture in the anastomosis area. The other patient initially had a mild narrowing of the anastomosis and subsequently required multiple dilations with no relief of the dysphagia. Once biopsies were taken from the esophagus and the diagnosis of EoE was made and treated, the symptoms did not reoccur. This suggests that the dilations were not adequate treatment in this patient, until the underlying diagnosis was made and treated effectively.

In conclusion, when presented with symptoms such as dysphagia later on in the life of a patient with EA repair, the diagnosis of EoE should seriously be considered and adequate biopsies should be taken prior to committing a patient to recurrent anastomotic dilatations. It is very important to consider the atopic history of these patients, especially when they begin to have complaints such as dysphagia. It is well known that asthma is more common in patients with esophageal atresia than in controls[9,29,30]. So the diagnosis of EoE should be highly suspected and is a logical association in patients. Through our experience we found that these patients' EoE is more difficult to treat. Likely a combination of their baseline poor motility due to the underlying EA along with EoE complicating the coarse. When a patient with a history of EA presents with dysphagia a diagnosis of EoE should be considered and further investigated.

COMMENTS

Case characteristics

Dysphagia is a common symptom among patients with a history of esophageal atresia, it can also be found concurrently with other esophageal conditions like Eosinophilic esophagitis.

Clinical diagnosis

Biopsies to confirm eosinophilic esophagitis (EoE) is an essential part of the evaluation of these patients and it allowed for different therapeutic options.

Differential diagnosis

Dysmotility, gastroesophageal reflux disease are among the other diagnosis that should be considered.

Laboratory diagnosis

Unfortunately there are no good laboratory testing methods to confirm the diagnosis of EoE in patients. Sometime peripheral eosinophilia or an elevated IgE might be present.

Imaging diagnosis

Imaging is an important part of evaluating patients, esophagrams can identify possible strictures.

Pathological diagnosis

Pathologic findings of greater than 15 eos/hpf are necessary to diagnose EoE

without associated eosinophilia in the stomach or duodenum.

Treatment

Once diagnosis of EoE is made, therapy should be aimed at treating the EoE, based on patient's atopic history, dietary restrictions can be an option, other times topical steroids are the best option.

Experiences and lessons

Biopsies are important to obtain in patients with the history of esophageal atresia who present with dysphagia. Treating strictures with dilation may not be an effective therapy if a patient has underlying EoE.

Peer review

Weakness is limited volume of patients, and underlying cause of EoE is unknown. Strength is outcome of the patients improved with the treatment of EoE.

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CASE REPORT

Signet-ring cell carcinoma arising from a fundic gland polyp in the stomach

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Abstract

Fundic gland polyps (FGPs) are currently the most common type of gastric polyps and are usually benign. However, although rare, gastric adenocarcinoma of FGP has been recently proposed as a new variant of gastric adenocarcinoma. Here we report the first case of a 49-year-old woman with focal signet ring cell carcinoma that arose from an FGP of the stomach. The tumor was completely excised by endoscopic snare polypectomy. FGPs should therefore be evaluated for malignant changes although they occur rarely, if the FGP has an erosive or irregular surface.

Key words: Fundic gland polyp; Signet ring cell

Core tip: We report the first case of a 49-year-old woman diagnosed with focal signet ring cell carcinoma that arose from a fundic gland polyp in the stomach. The tumor was detected and completely excised by endoscopic snare polypectomy. Although malignant transformation of fundic gland polyps (FGPs) is extremely rare, endoscopists should consider the association of gastric polyps with gastric cancer for both hyperplastic polyps and FGPs.

Jeong YS, Kim SE, Kwon MJ, Seo JY, Lim H, Park JW, Kang HS, Moon SH, Kim JH, Park CK. Signet-ring cell carcinoma arising from a fundic gland polyp in the stomach. *World J Gastroenterol* 2014; 20(47): 18044-18047 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/18044.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.18044

INTRODUCTION

Gastric fundic gland polyps (FGPs) are the most common lesions, and they have been regarded as benign. These polyps can be sporadic or associated with an inherited polyposis syndrome. Sporadic FGPs are typically regarded as benign lesions with no risk of malignant transformation. Some reports have described sporadic FGPs containing low-grade or high-grade dysplasia[1,2]. Adenocarcinoma of the fundic gland was recently proposed as a new variant of gastric adenocarcinoma[3,4]. However, there are no reports of signet ring cell carcinoma arising from a sporadic FGP. Herein, we report the case of a woman diagnosed with focal signet ring cell carcinoma originating from an FGP. The FGP with focal signet ring cell carcinoma was completely endoscopically excised by

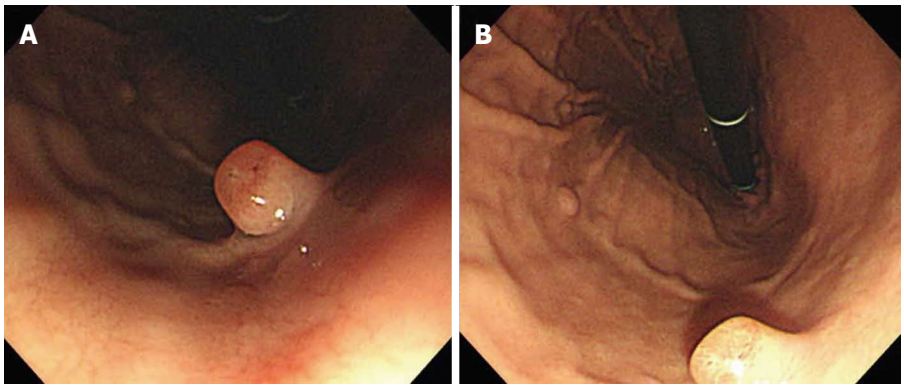

Figure 1 Endoscopic appearance of fundic gland polyps. A: The first polyp (0.8 cm in diameter, pedunculated, reddish, and with an erosive surface) at the mid body on the anterior wall; B: Other polyps (with a smooth surface) at the mid to high body on the greater curvature.

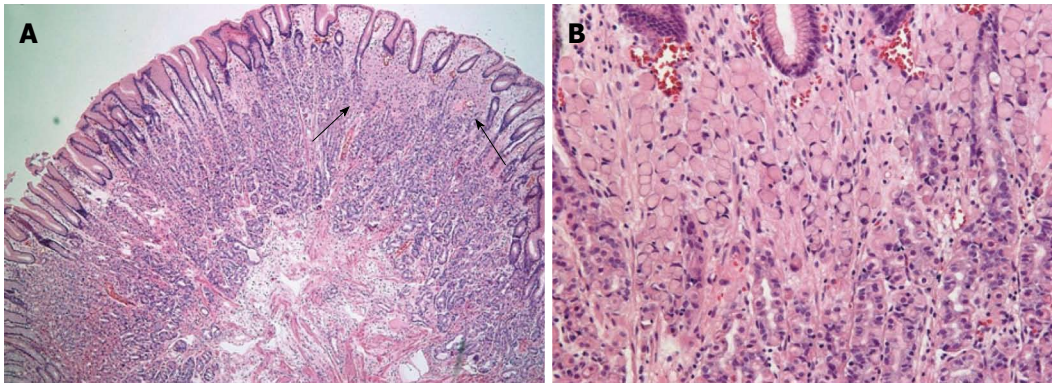

Figure 2 Histological findings. A: A resected specimen demonstrated signet ring cell carcinoma (between the arrows) (HE staining, × 40); B: Loosely cohesive signet ring cells from fundic oxyntic glands of fundic gland polyp infiltrate the lamina propria (HE staining, × 100).

snare polypectomy.

CASE REPORT

A 49-year-old woman was referred for gastric polyps in a mass-screening endoscopic examination at a local clinic. The patient did not describe any important past history data or complaints at admission. No specific familial history was identified. The physical examination and laboratory data were also unremarkable. Endoscopic examination of the upper digestive tract revealed four gastric polyps. The first polyp (0.8 cm in diameter, pedunculated, reddish, and with an erosive surface) was located at the mid body on the anterior wall (Figure 1A). The second and third polyps (0.5 cm in diameter, pedunculated, and with a smooth surface) were located at the mid body on the greater curvature. The fourth polyp (0.4 cm in diameter, pedunculated, and smooth) was at the high body on the greater curvature (Figure 1B). The first polyp was removed by snare polypectomy after epinephrine injection due to the erosive surface, even though it was small and suspected to be an FGP. The second, third and fourth polyps were removed using hot biopsy forceps. Histologically, the lesion consisted mainly of polypoid proliferation of fundic oxyntic glands, which were partially dilated and distorted, which is consistent with FGP. We also found a focus of loosely cohesive round cells that had a foamy eosinophilic cytoplasm and peripherally placed nuclei infiltrated lamina propria, measuring 0.2 cm × 0.1 cm, in the superficial portion of the polyp. The tumor cells were consistent with signet ring cell carcinoma, and they appeared to originate from fundic oxyntic cells of the FGP. The cancerous focus was confined within the mucosa (Figure 2). The tumor cells were positive for cytokeratin using immunohistochemistry. Approximately 80% of the tumor cells were positive for p53 protein (1:2000, clone DO-7, Novacastra, Newcastle upon Tyne, United Kingdom), while proliferative oxyntic cells of the FGP were negative (Figure 3). *Helicobacter pylori* (*H. pylori*) infection was negative. The second, third and fourth polyps were FGPs.

The first polyp was resected completely by snare polypectomy, and the resection margins were negative for carcinoma.

DISCUSSION

This novel case describes a signet ring cell carcinoma that originated from an FGP. The relationship between adenocarcinoma and gastric adenoma is well established[5]. However, gastric FGPs, which are the most common lesions, have typically been considered benign lesions with no risk

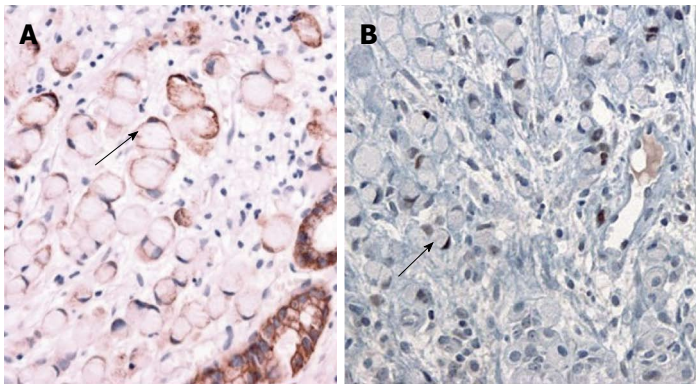

Figure 3 Immunohistochemical findings. A: Signet ring cell carcinoma is positive for cytokeratin (arrow); B: p53 is expressed in signet ring cell carcinoma but not in the fundic oxyntic cells (arrow).

of malignant transformation.

Sporadic FGPs are the most common type of gastric polyps that are found during upper endoscopy[6]. The prevalence of FGPs appears to be increasing due to the increased use of upper endoscopy, increased use of acid suppressive medications, and decreased prevalence of *H. pylori* infection[2].

The pathogenesis and malignant potential of FGPs are unknown. FGPs rarely occur before adulthood and 40%-50% spontaneously regress; both features are inconsistent with the traditional assumptions about hamartomas[7]. A case of dysplasia and adenocarcinoma has been documented in the FGPs of patients with attenuated adenomatous polyposis coli[8]. A female predominance in nonfamilial FGPs suggests that hormones play a role in the development of FGPs[9]. Some reports have described sporadic FGPs with low-grade or high-grade dysplasia[10,11] and adenocarcinoma[3,4,6]. There are no reports of gastric signet ring cell carcinoma arising from sporadic FGPs.

In the present case, the FGP (0.8 cm in diameter) was associated with a minute signet ring cell carcinoma and removed. The histologic differential diagnoses of signet ring cell carcinoma in gastric polyp included mucosal xanthelasma, clusters of mucin-laden macrophages in the lamina propria, and metastatic carcinomas composed of loosely cohesive cells, such as lobular carcinoma of the breast. In the present case, the tumor cells were positive for cytokeratin, which differentiates histiocytes from signet ring cell carcinoma. The present tumor cells were negative for the estrogen and progesterone receptors, and the systemic evaluation of the patient did not reveal any other malignancies.

Although the malignant transformation of FGPs is extremely rare, endoscopists should consider the association of gastric polyps and gastric cancer for both hyperplastic polyps and FGPs.

The polyp had a minute signet ring cell carcinoma that was removed using snare polypectomy due to the erosive surface, even if it was a suspected FGP. Histologically, the tumor cells were in the superficial portion of the polyp, but the relationship between the cancerous foci and the erosive surface was not clear. Little is known about the pathogenesis of and risk factors for the malignant transformation of FGPs. Inflammation or molecular alterations related to an erosive surface may be associated with the malignant transformation of FGPs.

Therefore, endoscopists should perform a thorough visual inspection of gastric FGPs. Any lesions, no matter how small, that appear significantly different from others (*e.g.*, erosive or irregular surface) should be biopsied or removed. When the surface of an FGP is eroded, the regenerative appearance can be interpreted as dysplasia; however, true dysplasia or malignant transformation of sporadic FGPs should be considered. Biopsies or endoscopically resected stomach specimens should be carefully examined microscopically for the possibility of malignancy.

In conclusion, we report the first case of a woman who was diagnosed with focal signet ring cell carcinoma originating from an FGP. This case shows that sporadic FGPs may also be related to gastric cancer. Although the association of FGPs and gastric cancer is not clearly established, biopsies or resection of FGPs, no matter how small, should be considered when the FGP has an erosive surface.

COMMENTS

Case characteristics

A 49-year-old woman with incidentally found gastric polyps.

Clinical diagnosis

Fundic gland polyp.

Differential diagnosis

Familial polyposis syndrome, malignancy.

Laboratory diagnosis

The laboratory datas were unremarkable.

Imaging diagnosis

Computed tomography scans of abdomen and pelvis were unremarkable.

Pathological diagnosis

Snare polypectomy revealed a focal signet ring cell carcinoma originating from a fundic gland polyp.

Treatment

The patient was not treated any further after snare polypectomy because the polyp was resected completely and the resection margins were negative for

carcinoma.

Related reports

Some reports have described sporadic fundic gland polyps (FGPs) containing low-grade or high-grade dysplasia. A gastric adenocarcinoma of the fundic gland was recently proposed as a new variant of gastric adenocarcinoma.

Term explanation

FGP means fundic gland polyp.

Experiences and lessons

This case report not only represents focal signet ring cell carcinoma in a FGP that have not been reported, but also provides a lesson that sporadic FGPs with different appearance from others should undergo a biopsy examination or, if possible, be removed.

Peer review

The direct histologic relationship between the cancerous foci and the erosive surface of FGP is not clear. But this case shows the possibility of malignant transformation in a sporadic FGP. This case can give endoscopists a lesson in the management of a sporadic FGP.

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CASE REPORT

Primary large cell neuroendocrine carcinoma in the common bile duct: First Asian case report

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Abstract

Large cell neuroendocrine carcinoma (LCNEC) in the biliary system is a poorly differentiated, high-grade neuroendocrine tumor. These tumors exhibit aggressive behavior and an increased tendency for early nodal and distant metastases. Herein, we report an unusual case of a pure primary LCNEC of the common bile duct (CBD). A 75-year-old female presented with nausea and jaundice. The patient underwent a CBD excision with lymph node dissection. Upon histological and immunohistochemical examination, the tumor exhibited pure large cell-type neuroendocrine features. Metastases were noted in two of the eight lymph nodes. The patient was administered adjuvant chemotherapy. The patient's cancer recurred 7 mo after surgery, and the patient died from liver failure 5 mo after recurrence. The prognosis of LCNEC of CBD remains poor despite curative resection and adjuvant chemotherapy. The role of additional therapies, such as multimodal treatment including radiation therapy, must be further studied to improve the prognoses of patients.

Key words: Neuro endocrine tumor; Large cell neuroendocrine carcinoma; Metastases; Multimodal treatment; Common bile duct

Core tip: Primary neuroendocrine carcinomas of the common bile duct rarely occur; among these tumors, large cell neuroendocrine carcinoma is extremely rare. Large cell neuroendocrine carcinoma (LCNEC) is a high-grade malignant neuroendocrine neoplasm. This case report represents a rare case of a primary neuroendocrine carcinoma of the common bile duct. More clinicopathological data and further studies with multimodal treatment are required to identify the prognostic indicators and histogenesis of LCNEC of common bile duct.

Park SB, Moon SB, Ryu YJ, Hong J, Kim YH, Chae GB, Hong SK. Primary large cell neuroendocrine carcinoma in the common bile duct: First Asian case report. *World J Gastroenterol* 2014; 20(47): 18048-18052 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i47/18048.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i47.18048

INTRODUCTION

Although neuroendocrine neoplasms are frequently observed in the gastrointestinal tract, primary neuroendocrine carcinoma (NEC) of the common bile duct (CBD) rarely occurs. Among these tumors, large cell NEC (LCNEC) is extremely rare. LCNEC is a high-grade malignant neuroendocrine neoplasm. LCNEC of the CBD was first described by Sato *et al*[1], and only two additional cases of

LCNEC of the CBD have been reported to date[2,3]. The pathogeneses of NEC of the CBD remains unclear. The diagnosis of CBD LCNEC is rarely made preoperatively because this tumor generally appears with nonspecific symptoms. Furthermore, the preoperative differentiation between cholangiocarcinoma and LCNEC is not possible using current imaging techniques. Histopathologically, reported LCNECs have been noted as pure forms or combined with adenocarcinoma or other tumors[2]. Given the invasive nature and aggressive biology of the tumor, the prognosis of patients with LCNEC of the CBD is poor[1]. Herein, we present a rare case of LCNEC of the CBD.

CASE REPORT

A 75-year-old female presented to our hospital with a 5-d history of nausea and jaundice. A physical examination revealed an icteric sclera, and the patient had neither abdominal tenderness nor a palpable mass in the right upper quadrant of the abdomen. She had hypertension and diabetes mellitus, and both were well controlled with medication. The patient had no family history of cancer. On the date of admission, the complete blood count and serum biochemical parameters were as follows: white blood cells, 10500 × 10^3/μL (3.8-10.0 × 10^3/μL); total bilirubin, 6.4 mg/dL (0.3-1.2 mg/dL); direct bilirubin, 5.6 mg/dL (0.1-0.5 mg/dL); aspartate aminotransaminase, 151 U/L (normal 15-41 U/L); alanine aminophosphatase, 302 U/L (14-54 U/L); aspartate transferase, 240 U/L (38-126 U/L); and gamma-glutamyltranspeptidase, 939 U/L (7-50 U/L). Carbohydrate antigen 19-9 (CA19-9) was 118.1 U/mL (0-37 U/mL). Carcinoembryonic antigen and alpha fetoprotein levels were within normal limits. The chest X-ray was within normal limits. An abdominal computed tomography (CT) scan revealed a mass that was approximately 2.7 cm in size located in the mid common bile duct and a regional metastatic node in the hepatoduodenal ligament (Figure 1). The endoscopic retrograde cholangiopancreatography demonstrated an asymmetric filling defect in the mid common bile duct (Figure 2). The cystic duct was well visualized. After the endoscopic sphincterotomy, an endoscopic nasobiliary drainage catheter was inserted. The patient underwent an operation with the presumed diagnosis of CBD cancer. The tumor of the bile duct was resected *via* cholecystectomy. The patient exhibited enlarged lymph nodes in the hepatoduodenal ligament and porta hepatis, which were also resected. No evidence of distant metastasis and organ invasion was noted. The resection margin of the proximal and distal bile duct frozen biopsy was tumor negative. Reconstruction was performed with a Roux-en Y hepaticojejunostomy. On histopathological examination, the tumor exhibited high cellularity and invasion throughout the entire CBD wall without serosal penetration. The tumor cells are monotonous, with relatively abundant cytoplasm and hyperchromatic nuclei that exhibited course chromatin and prominent nucleoli (Figure 3A, B). Immunohistochemical findings indicated

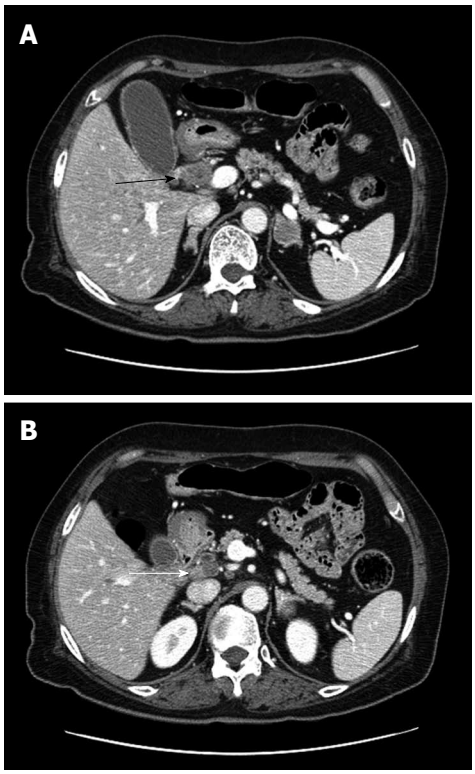

Figure 1 Computed tomography findings. A: One mass 2.7 cm in size in the mid common bile duct (black arrow); B: Regional metastatic node in the hepatoduodenal ligament (white arrow).

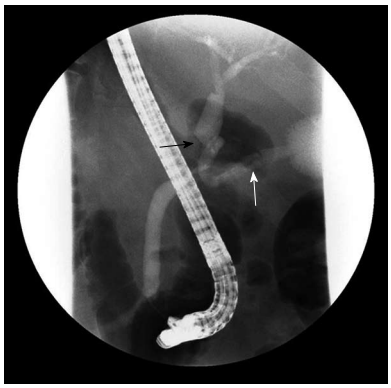

Figure 2 Endoscopic retrograde cholangiopancreatography. The image presents an asymmetric filling defect in the middle common bile duct with proximal dilatation (black arrow) and the cystic duct was well visualized (white arrow).

that tumor cells are immunopositive for neuroendocrine markers, including synaptophysin and chromogranin. No evidence of adenocarcinoma or other tumor components were noted (Figure 3C, D). Metastases were noted in two of eight lymph nodes. No postoperative complications occurred, and the patient was discharged. The patient was administered adjuvant chemotherapy consisting of 5-fluorouracil, epirubicin, and cisplatin. CT scans 7 mo after surgery indicated recurrence at the hepaticojejunostomy site as well as in liver and portocaval area. The patient died of liver failure five mo after recurrence.

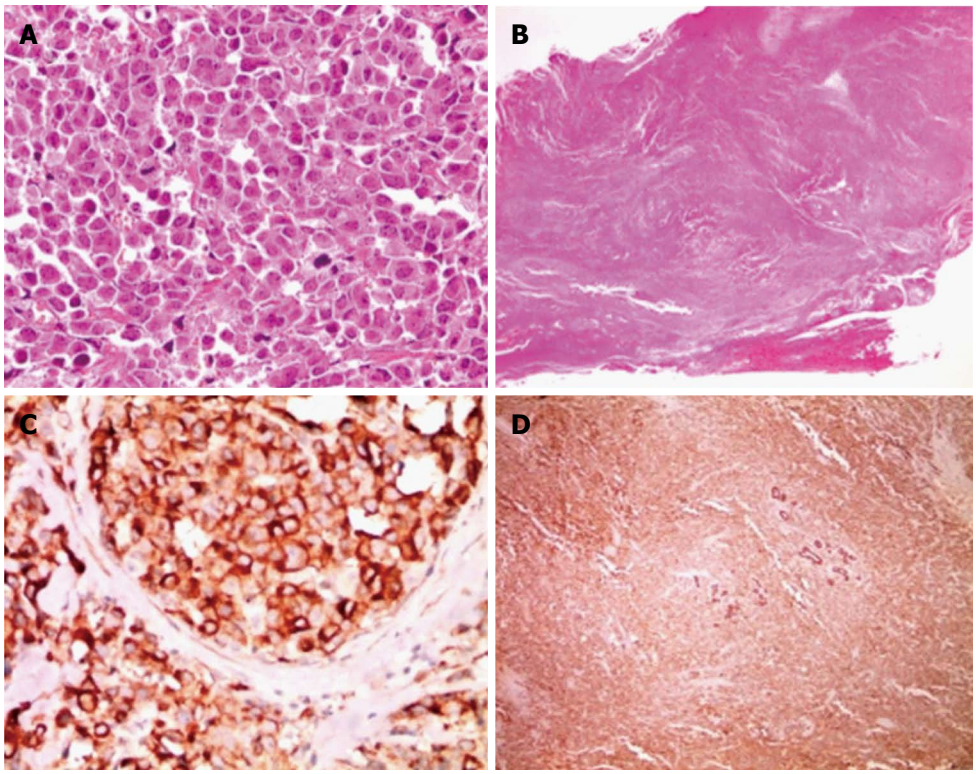

Figure 3 Histologic and immunohistochemical findings. A: High cellularity tumor cells invades into the bile duct wall (magnification × 12.5); B: tumor cells exhibit relatively abundant cytoplasm and hyperchromatic round nuclei with course chromatin and prominent nucleoli (magnification × 400); C: tumor cells exhibit immunopositivity for a neuroendocrine marker (synaptophysin) (magnification: × 400); D: immunohistochemical staining for Pan CK, and a combined adenocarcinoma component is not noted (magnification: × 40).

DISCUSSION

Carcinoma of the CBD accounts for less than 2% of all cancers, and the lesions identified in the CBD are predominantly cholangiocarcinomas (80%)[4]. The bile ducts is one of the rarest primary sites for neuroendocrine tumors (NETs), accounting for 0.2% to 2.0% of all such tumors[4]. Based on the WHO classification, neuroendocrine neoplasms are classified into five general categories, including NET, NEC, mixed adenoneuroendocrine carcinoma (MANEC), goblet cell carcinoid, and tubular carcinoid. In addition, NECs are classified as either LCNEC or small cell neuroendocrine carcinoma (SCNEC)[5]. Neuroendocrine neoplasms of the CBD are rare; carcinoid tumors represent the majority of these lesions.

A primary NEC of the CBD is extremely rare because normal CBD mucosa does not contain neuroendocrine cells. The origin of NEC of the CBD remains unclear. Neuroendocrine cells can be detected at sites of intestinal metaplasia induced by chronic inflammation, including long-standing chronic inflammation due to cholelithiasis and congenital anomalies, which may be the initial step in the development of neuroendocrine tumors of the CBD[6].

The symptoms of LCNEC of the CBD are non-specific. The preoperative diagnosis of LCNEC is difficult because it is not possible to differentiate LCNEC from an adenocarcinoma of the CBD using imaging studies, such as ultrasonography, CT, and abdominal angiography. Tumor markers, such as alpha fetoprotein, carcinoembryonic antigen, CA19-9 and CA125, are also non-specific.

LCNEC is a rare type of CBD, and to our knowledge, only three previous cases have been described in the English-language literature (Table 1)[1-3]. Thus, this is the fourth reported case of LCNEC occurring in the extra bile duct. The cases described by Sato *et al*[1] and Demoreuil *et al*[2] were MANECs (adenocarcinoma and LCNEC). Therefore, strictly speaking, our case is the second to report a pure LCNEC arising in the extra bile duct followed; the report of Sasatomi *et al*[3] was the first to report this type of lesion. Our study was the first report of an LCNEC in the CBD in an Asian individual.

More specific staining markers are required to accurately diagnose NECs of the CBD (*i.e.*, immunohistochemistry). The diagnosis of NEC should be confirmed by positive immunohistochemical staining for at least one neuroendocrine marker, such as chromogranin, synaptophysin, or neuron-specific enolase[7]. Microscopically, NET is defined as a well-differentiated neuroendocrine neoplasm with mild to moderate nuclear atypia and a low proliferation fraction (≤ 20 mitoses per 10 high-power fields or ≤ 20% Ki-67 index). Unlike NET, LCNEC is a poorly differentiated, high-grade malignant neuroendocrine neoplasm that is composed of large cells with marked nuclear atypia and a high proliferation fraction (> 20 mitoses per 10 high-power fields or > 20% Ki-67 index)[7].

Table 1 Reported cases of large cell neuroendocrine carcinomas of the common bile duct

Ref.	Sex/age (yr)	Tumor histology	Location size (cm)	Treatment	Survival duration (post OP)
Sato *et al*[1]	M/68	LCNEC + AD	D 2	Resection and chemotherapy	DD 3 mo
Demoreuil *et al*[2]	M/73	LCNEC + AD	P 3	Resection and chemotherapy	DD 12 mo
Sasatomi *et al*[3]	M/76	LCNEC	P 5	Resection	DD 21 d
Present report	F/75	LCNEC	M 3	Resection and chemotherapy	DD 12 mo

M: Male; F: Female; LCNEC: Large cell neuroendocrine carcinoma; AD: Adenocarcinoma; D: Distal common bile duct; P: Proximal common bile duct; M: Middle common bile duct; DD: Died of disease.

Therapeutic interventions have not been well defined, and no optimal postoperative adjuvant therapy, such as chemotherapy and radiation therapy, is available due to the rarity of LCNEC of the CBD and its poor prognosis. LCNEC is treated in a similar manner as other cancers in the CBD. The best treatment option is surgical excision. In most cases, adequate clearance can be achieved by an excision of the CBD *via* portal lymphadenectomy and Roux-en-Y hepaticojejunostomy reconstruction, but some distal CBD tumors are best treated by pancreaticoduodenectomy. The role of adjuvant therapy remains controversial, and most studies have failed to demonstrate a survival advantage[8].

The prognosis of patient with LCNEC of the CBD is reportedly poor[8]. The survival duration of previously reported LCNEC of the CBD cases ranged from only 21 d to 12 mo after surgery (Table 1, cases 1-3). In our case, recurrence was noted 7 mo after surgery, and the patient died 5 mo after this recurrence. SCNEC of the CBD, another NEC type, has a poor prognosis, similar to LCNES. Edakuni *et al*[9] reported the longest survival of SCNEC of the CBD as 45 mo after surgery. In this case, the tumor was a MANEC (40% adenocarcinoma and 60% SCNEC) and exhibited a reduced proliferative fraction (9.6% Ki-67-positive tumor cell). Shimono *et al*[10] reported a long survival (69 mo after surgery) in a gallbladder patient with LCNEC who underwent an operation and adjuvant chemotherapy as well as radiation therapy after surgery[10]. Though this case involves a single LCNEC of a gallbladder patient, the multimodal treatment appears to offer enhanced treatment, resulting in increased survival for LCNEC of the CBD.

LCNEC of the CBD is a poorly differentiated, rare tumor that exhibits high-grade NETs with aggressive behavior and has a high tendency for early lymph node and distant metastases. More clinicopathological data and further studies with multimodal treatment are required to identify the prognostic indicators and the histogenesis of LCNEC of the CBD.

COMMENTS

Case characteristics

A 75-year-old female presented with a history of nausea, increasing yellowish discoloration of the face and dark-colored urine.

Clinical diagnosis

Icteric sclera. The patient did not exhibit abdominal tenderness or a palpable mass in the right upper quadrant of the abdomen.

Differential diagnosis

Cholangiocarcinoma and other common bile duct (CBD) cancers.

Laboratory diagnosis

White blood cells, 10500 $10^3/\mu L$ (3.8-10.0 $10^3/\mu L$); serum albumin, 3.1 g/dL (3.5-4.8 g/dL); total bilirubin, 6.4 mg/dL (0.3-1.2 mg/dL); direct bilirubin, 5.6 mg/dL (0.1-0.5 mg/dL); aspartate aminotransaminase, 151 U/L (normal 15-41 U/L); alanine aminophosphatase, 302 U/L (14-54 U/L); aspartate transferase, 240 U/L (38-126 U/L); and gammaglutamyl transpeptidase, 939 U/L (7-50 U/L). The carbohydrate antigen 19-9 level was 118.1 U/mL (0-37 U/mL).

Imaging diagnosis

A computed tomography scan revealed a mass approximately 2.7 cm in size in the mid common bile duct and a regional metastatic node in the hepatoduodenal ligament. The endoscopic retrograde cholangiopancreatography demonstrated an asymmetric filling defect in the mid common bile duct.

Treatment

The patient underwent a CBD excision with lymph node dissection. The patient was administered adjuvant chemotherapy consisting of 5-fluorouracil, epirubicin, and cisplatin.

Related reports

Tumor cells are monotonous and exhibit relatively abundant cytoplasm and prominent hyperchromatic nuclei with course chromatin. Immunohistochemical findings indicated that tumor cells are immunopositive to neuroendocrine markers, including synaptophysin and chromogranin.

Experiences and lessons

This case report presents a rare case of a primary neuroendocrine carcinoma of the common bile duct. More clinicopathological data and further studies with multimodal treatment are required to identify the prognostic indicators and the histogenesis of large cell neuroendocrine carcinoma of the CBD.

Peer review

This article applies information about a rare case of a primary neuroendocrine carcinoma of the common bile duct.

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CASE REPORT

Acute pancreatitis associated with herpes zoster: Case report and literature review

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Abstract

Varicella-zoster virus (VZV) is a type of herpes virus known to cause varicella, mainly in young children, and herpes zoster in adults. Although generally non-lethal, VZV infection can be associated with serious complications, particularly in adults. Acute pancreatitis caused by VZV infection is a rare event, with reports primarily concerning immunocompromised individuals. Here we report a 44-year-old immunocompetent female who developed acute pancreatitis associated with VZV infection. The patient presented with vomiting and persistent pain in the upper quadrant less than one week after diagnosis and treatment for a herpes zoster-related rash with stabbing pain on the abdomen and dorsal right trunk side. A diagnosis of acute pancreatitis was confirmed based on abdominal pain, elevated levels of urine and serum amylase, and findings of peri-pancreatic exudation and effusions by computed tomography and magnetic resonance cholangiopancreatography. This case highlights that, though rare, acute pancreatitis should be considered in VZV patients who complain of abdominal pain, especially in the epigastric area. Early detection and proper treatment are needed to prevent the condition from deteriorating further and to minimize mortality.

Key words: Varicella-zoster virus; Herpes zoster; Acute pancreatitis; Immunocompetent adult

Core tip: Acute pancreatitis associated with varicella-zoster viral infection is extremely rare. This report presents the case of a 44-year-old woman who developed acute pancreatitis after the onset of herpes zoster. This is the first case report of acute pancreatitis associated with herpes zoster in an immunocompetent adult.

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INTRODUCTION

Decades after a primary infection, latent varicella-zoster virus (VZV) in the dorsal root ganglia of the sensory nerves[1] can reactivate and spread unilaterally along a dermatome to cause herpes zoster. Diagnosis is usually based on the characteristic varicella rash, which is vesicular, covers a single dermatome, and lasts for three to five days[2]. The most frequent site of reactivation is the ophthalmic division of the trigeminal nerve, which can involve the eyes and the thoracic nerves[2,3]. Without a typical rash, herpes zoster can also be confirmed by a virology laboratory or by testing for serum immunoglobu-

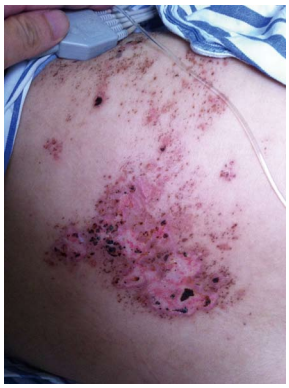

Figure 1 Presentation of characteristic rash. Image showing the rash, which had begun to scab, on the patient's right thoracodorsal area.

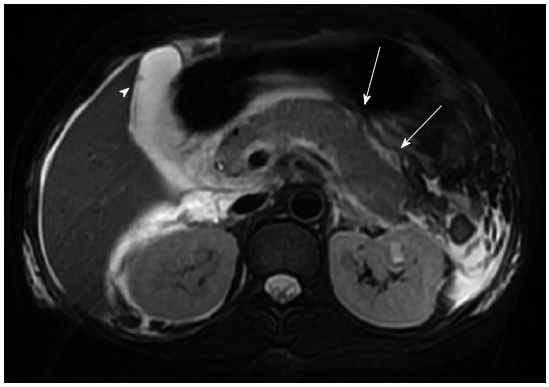

Figure 2 Magnetic resonance cholangiopancreatography. Image showing a punctiform low signal intensity in the gallbladder (arrowhead) and peri-pancreatic exudation (arrows).

lins M and A against VZV and the fluorescent antibody to membrane antigen test[2,4]. The most common complication is secondary bacterial infection, followed by other serious complications including pneumonia, encephalitis, myelitis, retinitis, hemiparesis, hepatitis and disseminated intravascular coagulopathy[4], which are more common in immunocompromised patients, such as transplant recipients and patients with acquired immune deficiency syndrome (AIDS). The occurrence of acute pancreatitis in association with VZV infection is very rare and has only been reported in immunocompromised individuals or children. Here, we present the first reported case of acute pancreatitis associated with VZV infection in an immunocompetent adult.

CASE REPORT

A 44-year-old woman experienced a pectoral and dorsal rash with persistent moderate stabbing pain on her right trunk. She was diagnosed with herpes zoster at a local hospital and treated with topical anti-viral drugs, which alleviated the pain. Five days later, the pain became worse after eating a regular meal, appearing in the epigastric area as well as the original location, and accompanied by vomiting. The pain was dull and severe, waking her in the night. Over the ensuing 48 h, she vomited approximately 400 mL of gastric content, with no fever or diarrhea present. At this time, the patient was admitted to the emergency department of our hospital. She had no significant past medical history, and denied any alcohol, drug or smoke consumption.

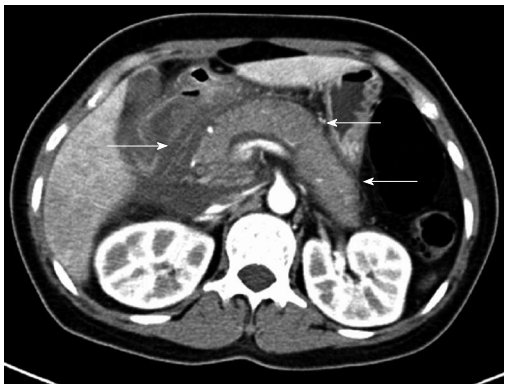

Figure 3 Contrast-enhanced computed tomography. Image showing the swelling of the pancreas with peri-pancreatic exudation and liquid collection (arrows). Grade E acute pancreatitis was diagnosed based on the computed tomography severity index.

On admission, physical examination showed a pulse rate of 107 beat/min, blood pressure of 113/71 mmHg, body temperature of 36.9 ℃, and a respiration rate of 19 breaths/min. Pulse oximetry showed a normal (97%) O_2 saturation. Moderate tenderness in the upper abdomen was observed with no rebound tenderness, a rectal examination was normal, and heart and chest auscultation did not reveal any findings. No jaundice was seen in the skin and sclera. A sheet-like rash was noted in the right thoracodorsal area (Figure 1). Laboratory analysis of blood tests showed elevations of many proteins (Table 1). Magnetic resonance cholangiopancreatography revealed peri-pancreatic exudation and a punctiform low signal intensity in the gallbladder (Figure 2), which was identified as a small cholecystic polyp after additional ultrasound examination. Abdominal contrast-enhanced computed tomography (CT) showed acute pancreatitis (American Roentgen Ray Society severity index of 6[5], Balthazar stage E[6]) with swelling of the pancreas, peri-pancreatic exudation and liquid collection (Figure 3). The combined results indicated moderately severe acute pancreatitis according to the revised Atlanta classification[7] and a Ranson score of 4[8]. The decreased serum calcium concentration and elevated blood glucose also indicated significant impairment of the pancreas with a poor prognosis.

The patient was fasted upon admission and received passive gastric decompression along with mask oxygen inhalation, fluid resuscitation, and total parenteral nutrition. She was administered octreotide (0.05 mg/h, iv), lansoprazole (30 mg b.i.d.), antibiotics (imipenem and cilastatin sodium, 0.5 g q8h, iv), an analgesic (dezocine) as occasion required, and a subcutaneous insulin injection for hyperglycemia. Vomiting ceased on the first day

Table 1 Laboratory findings of the patient

Testing items	Results	Reference range
WBC, count/mL	12800	4000-10000
HCT, %	43.20	33.5-45.0
serum amylase, IU/L	456	20-80
urine amylase, IU/L	10999	42-321
CRP, mg/L	214.7	< 10
c(Ca^{2+}), mmol/L	1.63	2.08-2.60
Alb, g/L	34.9	35.0-52.0
LDH, U/L	539	140-271
FBG, mmol/L	14.73	3.89-6.11
HbA1C, %	6.20	4.3-6.3
ESR, mm/h	46	< 20
HIV	(-)	(-)
HBsAg	(-)	(-)
HCVAb	(-)	(-)

Alb: Serum albumin; c(Ca^{2+}): Serum calcium concentration; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; FBG: Fasting blood glucose; HbA1C: Glycosylated hemoglobin; HCT: Hematocrit; HBsAg: Hepatitis B surface antigen; HCVAb: Hepatitis C virus antibody; HIV: Human immunodeficiency virus; LDH: Lactic dehydrogenase; WBC: White blood cell.

of admission, abdominal pain gradually improved, and the rash progressively regressed. The herpes zoster rash was determined by a dermatologist to be in the recovering phase and locally treated with acyclovir ointment. The patient began enteral nutrition on the tenth day after amylase levels decreased to normal and C-reactive protein levels had decreased noticeably. She was permitted a fluid diet after 20 d, and discharged 27 d after admission with normal serum chemistry work-up results. The patient remained well during the one-month follow-up.

DISCUSSION

Acute pancreatitis is an inflammatory disease of the pancreas that may also involve peri-pancreatic tissues. The most common causes of acute pancreatitis in adults are common bile duct stones and alcohol abuse, accounting for 38% and 36% respectively, though other etiologies include toxins, drugs, surgery, and metabolic or autoimmune conditions, as well as infections (from bacteria, mycoplasma, virus and parasites)[9]. The most common symptom of acute pancreatitis is acute abdominal pain, especially in the epigastrium, as well as gastrointestinal symptoms such as nausea and vomiting. Imaging results and increased concentrations of serum and urine amylase (exceeding three times the normal upper limit) and lipase can be used to directly diagnose 81%-95% of patients[10].

Of the few cases of herpes zoster-associated acute pancreatitis that have been reported, most involve immunocompromised individuals, such as those in intensive care[11], AIDS patients[12], or receiving long-term immunosuppression, such as recipients of stem cell[13-15], renal[16,17] or liver[18] transplants. Three studies reported acute pancreatitis associated with VZV in children with chickenpox[19-21] and one study reported an elderly patient with herpes zoster suffering from systemic complications including pancreatitis and encephalitis[22]. The case presented here is the first report of acute pancreatitis in an immunocompetent adult without comorbidity. Although the mechanism of pancreatitis with herpes zoster is still unknown, VZV may remain latent in posterior sensory nerve roots that contain fibers from both skin and abdominal viscera, including the pancreas[23]. VZV might injure the pancreatic acinar cell membrane, leading to the leakage of intracellular enzymes, or the cytopathic effect may be mediated through the patient's immune response.

In the present case, the patient's pain initially began as a constant, stabbing pain at the site of the rash on the abdomen. Although the location and the quality of the pain changed several days later, it could have easily been attributed to neuralgia caused by herpes zoster and ignored by mistake. The severity of the nausea and vomiting prompted the patient to seek additional treatment. At this time, the diagnosis of acute pancreatitis was not difficult to confirm, based on serum amylase elevation and CT imaging. Although the etiology is not evident, the influence of VZV on the pancreas gained attention, as common contributors, such as cholelithiasis, alcoholism, common infection and immune deficiency, were excluded. However, the management of viral acute pancreatitis is comparable to acute pancreatitis caused by other etiologies, involving supportive treatment with electrocardiograph monitoring, fluid resuscitation, pain control, and fasting with temporary enteral or total parenteral nutrition.

In conclusion, although rare, this case highlights the need to consider acute pancreatitis as a differential diagnosis for immunocompetent patients with herpes zoster, particularly when the location and quality of the pain changes during the course of the disease. Early detection and proper treatment is needed when acute pancreatitis is suspected.

COMMENTS

Case characteristics

A 44-year-old woman presented with persistent pain in the upper quadrant and vomiting less than one week after the onset of a rash with stabbing pain, characteristic of varicella, on the abdomen and dorsal right trunk.

Clinical diagnosis

Abdominal pain, elevated level of urine and serum amylase concentration, computed tomography findings of peri-pancreatic exudation and effusions.

Differential diagnosis

Common etiologies for acute pancreatitis were excluded based on the absence of alcohol, drug or smoke consumption. Computed tomography and magnetic resonance cholangiopancreatography (MRCP) showed no evidence of gallstones or pancreatic tumor.Laboratory findings did not indicate hypercalcemia or hyperlipidemia.

Laboratory diagnosis

Serum amylase, 456 IU/L; urine amylase, 10999 IU/L; C-reactive protein, 214.7 mg/L; calcium concentration, 1.63 mmol/L; lactic dehydrogenase, 539 U/L.

Imaging diagnosis

MRCP and contrast-enhanced CT showed acute pancreatitis with swelling of the pancreas, peri-pancreatic exudation and liquid collection.

Treatment

The viral acute pancreatitis was managed with supportive treatment, including electrocardiograph monitoring, fluid resuscitation, pain control, and fasting with temporary enteral nutrition. The patient also received octreotide, lansoprazole,

antibiotics, and an analgesic for the herpes zoster, and a subcutaneous insulin injection for hyperglycemia.

Related reports

Reports of acute pancreatitis associated with varicella-zoster virus (VZV) are rare, with only three previous studies involving children with chickenpox, and one study reported involving an elderly patient suffering from systemic herpes zoster-related complications including pancreatitis and encephalitis.

Term explanation

Herpes zoster refers to the reactivation of a latent VZV.

Experiences and lessons

This literature review highlights the need to consider acute pancreatitis in the differential diagnosis when the location and quality of a patient's pain changes during the course of varicella disease.

Peer review

This interesting case is the first report of acute pancreatitis associated with VZV infection in an immunocompetent adult. The authors describe the clinical features, physical examination, laboratory findings, and MRCP and CT imaging in this case. Although the etiology of viral acute pancreatitis is unknown, suspected cases should be confirmed and treated as early as possible.

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Helicobacter, gamma-glutamyltransferase and cancer: Further intriguing connections

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Abstract

Virulence of *Helicobacter pylori*, *Helicobacter suis* and other bacteria appears to be partly mediated through a release of gamma-glutamyltransferase (GGT), an enzyme activity capable of promoting biochemical reactions ultimately resulting in damage to gastric epithelium and suppression of immune response. Recently published studies show that secretion of bacterial GGT occurs in the form of exosome-like vesicles. Very similar GGT-rich exosomes have been described to originate from human cancer cells, and the hypothesis is thus forwarded that in the resistant and invasive phenotype of malignant cells such vesicular/exosomal GGT may play roles akin to those described for *Helicobacter* infection, thus providing a significant contribution to the establishment of cancer metastases.

Key words: *Helicobacter pylori*; *Helicobacter suis*; Virulence; Gamma-glutamyltransferase; Immunosuppression; Cancer metastasis

Core tip: Biochemical reactions promoted by gamma-glutamyltransferase (GGT) of *Helicobacter* is capable of causing damage to gastric epithelium and suppression of immune response. Bacterial GGT is secreted as exosome-like vescicles, and very similar GGT-rich exosomes are released from human cancer cells. In the resistant and invasive phenotype of malignant cells, such secreted GGT may play roles akin to those described for *Helicobacter* infection, concurring to the establishment of cancer metastases.

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TO THE EDITOR

The recent Topic Highlight article by Ricci *et al*[1] is a thorough and comprehensive scrutiny of the mechanisms possibly underlying the recently reported implication of *Helicobacter pylori* (*H. pylori*) gamma-glutamyltransferase (GGT) as a virulence factor. The authors discuss how-among other effects-*H. pylori* GGT causes glutathione consumption and reactive oxygen species generation in the host cells, thus causing in turn cell-cycle arrest, apoptosis, and necrosis in gastric epithelial cells. Importantly, GGT also induces immune tolerance through the inhibition of T cell-mediated immunity and dendritic cell differentiation, overall favouring *H. pylori* persistence and gastric colonization[1].

Now, secretion of GGT appears to also occur by other bacteria related with gastric diseases, *e.g.*, *Campylobacter jejuni*[2], and recent reports are adding intriguing observations. Zhang *et al*[3] have shown that GGT of *H.*

suis-a related Helicobacter, also involved in gastric pathology - is secreted in the form of bacterial outer membrane vesicles (OMV), *i.e.*, submicroscopic structures 20-50 nm in diameter normally budding from the cell surface. These OMV's can translocate across the epithelial layers and deliver GGT enzyme to the lymphocytes residing in the lamina propria of gastric mucosa. As a result, inhibition of lymphocyte proliferation is induced, and bacterial invasion and proliferation are facilitated.

The association of bacterial GGT enzyme with OMV's may be the factor determining its targeting to host lymphocytes, but additional studies are needed to verify this point. In any case, one aspect of the matter calling for attention is represented by the connections possibly linking these observations with data stemming from oncologic research. Interestingly enough, secretion of similar GGT-containing submicroscopic particles has in fact been documented also from eukaryotic cells, and remarkably, from human cancer cells. GGT activity is expressed in a number of human malignancies, and increasing levels are usually detectable along with progression of the disease and in metastases[4,5]. GGT activity of cancer cells can affect intracellular redox equilibria, along with modulatory effects on the S-thiolation status of extracellular proteins[6], including cell surface receptors related with the cell survival/apoptosis balance[7]. Recent studies from our laboratory[8,9] have shown that active GGT can be released from cancer cells in association with vesicles similar to exosomes, 20-40 nm in diameter. The resemblance of such structures with GGT-rich OMV particles of *H. suis* is indeed obvious.

Thus, in the light of the mentioned studies on the virulence of *H. pylori,* the intriguing hypothesis could be forwarded that GGT-rich exosomes released by cancer cells can produce-in host's surrounding tissues-effects comparable to those apparent for bacterial GGT, *i.e.*, depletion of glutathione, oxidative stress and perturbation/suppression of immune response. This could contribute significantly to the increased ability of malignant cells to survive and colonize host's tissues, thus at least partially explaining the reportedly higher metastatic potential of GGT-expressing tumors[4]. The potential role in tumor evolution of GGT released by gastric cancer cells has not been investigated to date. Nevertheless, basing on the considerations above, clinical studies specifically addressing this point are warranted.

In conclusion, the GGT-dependent processes documented in bacterial virulence as well as in biology of malignant tumors may represent an example of "convergent evolution"-in unrelated species, and in different cells of the same species-of closely related molecular strategies, aimed at improving the survival and expansion of cellular populations in the context of a hostile/resisting environment. Future investigation will hopefully further elucidate these fascinating phenomena.

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Hepatocellular carcinoma review: Current treatment, and evidence-based medicine

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Abstract

We read with great interest the recent article entitled "Hepatocellular carcinoma review: Current treatment, and evidence-based medicine" by Raza *et al*, published in *World Journal of Gastroenterology*. Authors evaluated treatments for early and advanced stage hepatocellular carcinoma based on an extensive review of the relevant literature. They reported that radiofrequency ablation is the most effective local ablative therapy. They concluded that RF ablation is equivalent to surgical resection in well selected patients with early stage hepatocellular carcinoma. In addition, we want to mention microwave ablation besides RF ablation.

Key words: Hepatocellular carcinoma; Microwave ablation; Radiofrequency ablation

Core tip: We read with great interest the recent article entitled "Hepatocellular carcinoma review: Current treatment, and evidence-based medicine" by Raza *et al*, published in *World Journal of Gastroenterology*. Authors evaluated treatments for early and advanced stage hepatocellular carcinoma based on an extensive review of the relevant literature. They reported that radiofrequency ablation is the most effective local ablative therapy. They concluded that radiofrequency ablation is equivalent to surgical resection in well selected patients with early stage hepatocellular carcinoma. In addition, we want to mention microwave ablation besides RF ablation.

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TO THE EDITOR

We read with great interest the recent article entitled" Hepatocellular carcinoma review: Current treatment, and evidence-based medicine" by Raza *et al*[1], published in *World Journal of Gastroenterology*. Authors evaluated treatments for early and advanced stage hepatocellular carcinoma (HCC) based on an extensive review of the relevant literature. Authors reported that radiofrequency ablation (RF) is the most effective local ablative therapy. They concluded that RF ablation is equivalent to surgical resection in well selected patients with early stage HCC. In addition, we want to mention microwave ablation besides RF ablation. Unfortunately RF ablation use is limited by difficulties in heating charred tissue and has poor success rates for tumors near blood vessels, which is called heat-sink effect. Such limitations to heating can lead to potentially inadequate ablation zone and a higher rate of local tumor progression compared with resection[2]. Microwave ablation can heat the tissue faster than RF, and heating occurs in a large volume around the applicator. It would produce higher intratumoral temperatures, larger ablation zones, less ablation time and less dependence on the electrically conductive tissue. Its energy delivery is less limited by the exponentially rising electrical impedance of tumor tissue. These advantages may make microwave ablation less affected by heat-sink[3]. The advantage of RF is still and it

has been considered the most common thermal ablation modality worldwide for early stage HCC. Shi *et al*[4] reported that for solitary HCC ≤ 3 cm, MWA is as effective as surgical resection. In another report it is concluded that both MWA and RFA are safe and effective ablative treatments for liver cancer. Additionally, MWA has the advantage of cost efficiency[5]. On the other hand it is reported that there were no significant differences in morbidity or survival based on the surgical approach; however, local recurrence rates were highest for percutaneously ablated tumors[6].

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Statistical data

Write as mean ± SD or mean ± SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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