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EDITORIAL

P-glycoprotein multidrug transporter in inflammatory bowel diseases: More questions than answers

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Abstract

The gastrointestinal barrier is constantly exposed to numerous environmental substrates that are foreign and potentially harmful. These xenobiotics can cause shifts in the intestinal microbiota composition, affect mucosal immune responses, disturb tissue integrity and impair regeneration. The multidrug transporter *ABCB1/MDR1* p-glycoprotein (p-gp) plays a key role at the front line of host defence by efficiently protecting the gastrointestinal barrier from xenobiotic accumulation. This Editorial discusses how altered expression and function of *ABCB1/MDR1* p-gp may contribute to the development and persistence of chronic intestinal inflammation in inflammatory bowel diseases (IBD). Recent evidence implies multiple interactions between intestinal microbiota, innate immunity and xenobiotic metabolism *via* p-gp. While decreased efflux activity may promote disease susceptibility and drug toxicity, increased efflux activity may confer resistance to therapeutic drugs in IBD. Mice deficient in MDR1A develop spontaneously chronic colitis, providing a highly valuable murine IBD model for the study of intestinal epithelial barrier function, immunoregulation, infectious co-triggers and novel therapeutic approaches. Possible associations of human *ABCB1* gene polymorphisms with IBD susceptibility have been evaluated, but results are inconsistent. Future studies must focus on further elucidation of the pathophysiological relevance and immunological functions of p-gp and how its ambiguous effects could be therapeutically targeted in IBD.

Key words: Inflammatory bowel diseases; Multidrug resistance; Innate immunity; Microbiota; Xenobiotics

Core tip: Altered levels of p-glycoprotein (p-gp) expression as well as genetic variants of *ABCB1/MDR1* have been associated with inflammatory bowel diseases (IBD). Decreased efflux activity of p-gp may promote disease susceptibility, while increased efflux activity may impair drug responses in IBD. In this Editorial, I highlight what we need to know about this transporter

and xenobiotic signaling pathways in order to better understand its potential pathophysiology in IBD and develop targeted therapies.

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INTRODUCTION

The gastrointestinal (GI) barrier is constantly exposed to numerous environmental substrates that are foreign and potentially harmful, so-called xenobiotics. Toxic compounds can cause shifts in the intestinal microbiota composition, affect host innate and adaptive immune responses, disturb tissue integrity and impair regeneration. Several dysfunctions in xenobiotic recognition and metabolism have previously been implicated in the pathogenesis of inflammatory bowel diseases (IBD)[1-3]. To maintain mucosal homeostasis and prevent immunotoxic effects of xenobiotics, the GI barrier is equipped with a variety of detoxification mechanisms, including efflux transporters.

This Editorial focuses on recent insights into the *ABCB1/MDR1* (*multi-drug resistance*) - encoded p-glycoprotein (p-gp), which represents the most investigated ATP-dependent efflux transporter pump of xenobiotics (including metabolic products, toxins and drugs) in the intestine, and its impact on IBD pathophysiology. Growing evidence implies that altered expression and function of *ABCB1/MDR1* p-gp may contribute to the development and persistence of chronic intestinal inflammation in IBD. While decreased efflux function may mediate disease susceptibility and trigger drug toxicity, increased efflux activity may confer resistance to drug therapy in IBD.

STRUCTURE AND FUNCTION

P-gp, cloned in 1985[4], was initially described as a control mechanism of drug permeation and release at the membrane surface of colchicine-resistant Chinese hamster ovary cells[5]. In humans, the drug transporter p-gp is encoded by the *ABCB1/MDR1* gene (located on chromosome 7q21), while in rodents, p-gp is encoded by two genes, *Abcb1a/Mdr1a* and *Abcb1b/Mdr1b*. The N-terminal glycosylated protein consists of 1280 amino acids with a molecular mass of approximately 170 kDa. Murine p-gp shares 87% amino acid sequence identity with the human homologue[6], which makes knockout (KO) mouse models useful to study.

The secondary structure of *ABCB1/MDR1* p-gp contains two symmetrical halves of an ATP-binding domain (also known as "nucleotide binding domain") in the cytoplasm and a transmembrane domain with six hydrophobic α-helices, which are separated by a highly charged "linker region"[7,8]. Its transport activity depends on energetic metabolism and ATP hydrolysis. Once a substrate gets captured within the internal cavity of p-gp, ATP binds to its domains which causes a large conformational change presenting the substrate und drug-binding site to the extracellular space[6]. Thus, p-gp efficiently detoxifies cells by exporting hundreds of chemically and pharmacologically unrelated substances, including many important IBD drugs, such as steroid hormones (glucocorticosteroids), immunosuppressive agents (cyclosporine, tacrolimus), antimetabolites (methotrexate) or antibiotics (levofloxacin), and metabolic products. In addition, p-gp may also be involved in the transmembrane transport of pro-inflammatory cytokines, such as interleukin (IL)-2 and interferon-gamma (IFN-γ)[9], however, it remains to be shown how cytokine release could be directly regulated by p-gp signaling.

DIFFERENTIAL REGULATION OF EXPRESSION

The basal expression pattern of p-gp shows high inter- and also intraindividual variability along the GI tract, with a general increase from proximal to distal parts[10]. While *ABCB1/MDR1* p-gp is constitutively expressed at the frontline of the mucosal barrier, *i.e.* at the apical pole of intestinal epithelial cells, it is also inducibly expressed by many other cell types (*e.g.* macrophages[11] and T cell subsets[12]) in the lamina propria.

P-gp expression and function can be modulated by numerous exogenous and endogenous factors - based on the activation state of the individual cell and influences of its surrounding environment. Innate and adaptive immune responses, oxidative or inflammatory stress, dietary antigens, gut microbiota and other environmental triggers may differentially influence host metabolic signaling and xenobiotic transport *via* p-gp in the intestinal mucosa. The human *ABCB1/MDR1* promoter region contains multiple transcription factor-binding sequences, including specificity protein 1 (Sp-1), activator protein 1 (AP-1), nuclear factor interleukin-6 (NF-IL-6), forkhead transcription factor (FKHR) or T-cell factor/lymphoid enhancer factor (TCF/LEF), which points to complex regulation[13]. Upstream, the nuclear pregnane X receptor (PXR) may control convergence between xenobiotic detoxification and innate immunity by modulating transcription of p-gp as well as activation of (NACHT-, LRR- and PYD-containing Protein 3 (NLRP3)[14] and Toll-like receptor 4 (TLR4) signaling[15].

Downregulation of p-gp expression has been associated with acute intestinal inflammation, such as in the experimental mouse model of dextran sulphate sodium (DSS)-induced colitis[16] or in some patients with active

ulcerative colitis (UC)[17]. Increased mucosal levels of tumor necrosis factor alpha (TNFα) in active IBD suppress gene transcription of *ABCB1/MDR1* in intestinal epithelial cells, thus impairing xenobiotic efflux *via* p-gp[18]. Other major cytokines in IBD, such as IL-1ß or IL-6[19], may also interfere with p-gp expression and function. Interestingly, rifaximin, a non-absorbable antibiotic potentially beneficial for inducing remission in Crohn's disease (CD)[20], may antagonize TNFα-induced inhibition of p-gp *via* PXR[21].

Varying levels of p-gp in the intestinal mucosa may also be attributed to circadian rhythms caused by clock gene products which - at least in part - control *ABCB1/MDR1* gene expression[22]. Circadian expression of p-gp in the intestine may functionally affect the pharmacokinetics of its substrates, leading to temporal changes in intestinal absorption and excretion[23]. Of note, changes in the expression of several circadian genes have also been observed in active IBD[24]. Future research is needed to clarify the potential role of IBD-related circadian alterations in disturbing xenobiotic metabolism *via* p-gp.

MDR1A KO MOUSE MODEL OF SPONTANEOUS CHRONIC COLITIS

Mice deficient in MDR1A, first described by Dr. Alfred Schinkel in 1994, have initially been shown to be highly sensitive to the pesticide ivermectin and the chemotherapy drug vinblastine due to a blood-brain barrier defect[25]. Few years later, Dr. Jo Viney's group observed that MDR1A KO mice develop spontaneously chronic colitis that resembles human UC in several histopathological features[26,27]. Since then, numerous reports have proven that MDR1A KO colitis provides a highly valuable murine IBD model for the study of intestinal epithelial barrier function[28,29], immunoregulation[30-32], infectious co-triggers[33-35], and novel therapeutic approaches[36,37].

Typically, MDR1A KO pancolitis involves massive inflammatory thickening of the mucosa, increased crypt length with occasional abscesses, and goblet cell loss[26,27]. MDR1A KO colitis is driven by aberrant Th1 cytokine responses, associated with increased numbers of infiltrating CD4+ and TCRαβ+ T cells to the lamina propria[26] and intraepithelial lymphocyte alterations[30]. Dr. Robin Lorenz' group has recently shown that MDR1A KO mice display decreased numbers of CD4+Foxp3+ regulatory T cells in intestinal lymphoid tissues prior to the onset of disease, implying a primary defect in mucosal immunoregulation in the context of MDR1A deficiency[32].

Based on the MDR1A KO colitis model, it has been proposed that mechanisms involving mucosal upregulation of p-gp expression and/or function could have therapeutic potential in ameliorating acute IBD. Examples of potential p-gp inducers are listed in[38]. In addition, administration of Keratinocyte Growth Factor 2 or probiotics leads to increased mucosal p-gp expression[39,40], which is associated with attenuation of acute intestinal inflammation[41,42]. Future research must provide functional proof that upregulation of p-gp directly contributes to anti-inflammatory effects in the intestine.

INTERPLAY WITH GUT MICROBIOTA

The gut microbiome is involved in the pathogenesis of IBD. Tolerance to bacterial antigens is broken in active IBD and alterations in gut microbiota diversity contribute to inflammation and effector immune responses[43]. Several lines of evidence link gut microbiota and xenobiotic metabolism[44] *via* p-gp in the intestine.

Intestinal inflammation in MDR1A KO mice is commensal microbiota-dependent. MDR1A KO mice housed under germ-free conditions do not develop colitis[28] and oral antibiotic treatment significantly ameliorates disease[26,36]. Although commensal-mediated spontaneous colitis of MDR1A KO is not transmissible to wild-type animals[26], disease is exacerbated by infection with various pathogens, including bacteria (*e.g.*, *Helicobacter bilis*[33]), viruses (*e.g.*, murine norovirus[35]) or parasites (*e.g.*, *Trichuris muris*[45]). Animal feed, often contaminated by bacterial antigens[46], may also aggravate intestinal inflammation in MDR1A KO mice[47].

Intestinal p-gp limits bacterial invasion and dissemination. For instance, overexpression of p-gp in intestinal epithelial cells leads to increased resistance to *Listeria monocytogenes* or *Salmonella typhimurium* infection[48,49], while mice deficient in MDR1A exhibit enhanced burden of *Listeria monocytogenes* as compared to wildtype after infection[48]. But it remains unclear whether p-gp is capable of directly expelling virulence factors and toxins of bacterial pathogens from host cells. Signaling *via* p-gp might also fight infection by activating distinct immune processes, such as inducing production of type Ⅰ interferon in response to *Listeria monocytogenes*[50].

Dysbiosis precedes the onset of overt colonic inflammation in MDR1A KO mice[51], allowing certain, yet unknown, microbial species to colonize and expand. Lack of p-gp causes intestinal epithelial cell and barrier defects[28,29,52], leading to increased permeability and bacterial translocation which may induce excessive innate immune activation in the underlying lamina propria. Enhanced lipopolysaccharide signaling *via* MD-2/TLR4 in the intestinal mucosa seems to be required for perpetuation of colitis in MDR1A KO mice[36]. It remains to be tested whether genetic deficiency of MDR1A primarily determines changes in the microbial composition, or rather secondarily subverts the host innate immune response for creating an aberrant mucosal microenvironment that favours microbial misrecognition and shifts.

One may also speculate that impaired efflux pump activity in MDR1A deficiency could lower the threshold

for dysbiosis and pro-inflammatory conditions by accumulation of harmful xenobiotic compounds and metabolites in the intestinal mucosa. Xenobiotics and their metabolites may shape the complex dynamics of the gut microbiome[53] by providing substrates for selective growth of certain bacterial species, modulating gene expression[54], breaking microbial tolerance and triggering immune hypersensitivity to otherwise harmless commensals in the intestinal mucosa. Future studies must identify unremoved xenobiotic metabolites in intestinal MDR1A deficiency, examine their environmental effects on the microbiome and the mucosal immune system and analyse how they may contribute to colitis development.

Conversely, gut microbiota may directly affect host xenobiotic metabolism and detoxification by modifying p-gp signaling in the intestinal mucosa[53]. For instance, pathogenic *Salmonella typhimurium* dampens p-gp expression in intestinal epithelial cells[49]. So far, it is unclear which virulence factors or components of *Salmonella typhimurium* may be involved in impairing host p-gp function[55]. On the other hand, commensal *Lactobacilli* strains are capable of stimulating p-gp expression *via* the involvement of c-Fos/c-Jun in intestinal epithelial cells[40,56]. It is likely that this effect is mediated by TLR2 activation, as TLR2 signaling, which is induced by *Lactobacillus*[57,58], modulates ABCB1/MDR1-encoded p-gp synthesis and efflux function in intestinal cells[59]. Interestingly, deletion of TLR2 causes fulminant exacerbation of pancolitis in the context of MDR1A deficiency[36]. TLR2/MDR1A double KO intestinal myeloid cells hyperrespond to non-pathogenic *Escherichia coli* with excessive cellular stress, including increased reactive oxygen species generation, associated lysosomal damage and caspase-1-dependent IL-1ß production, leading to pyroptosis - a form of microbial-induced pro-inflammatory cell death[36]. Blockade of IL-1β activity by treatment with IL-1R antagonist (anakinra) inhibits colitis acceleration in TLR2/MDR1A double deficiency[36]. These data uncover an unexpected combinatory function between host innate immunity (TLR2) and xenobiotic metabolism (MDR1A) in controlling antimicrobial host defence in the lamina propria.

Taken together, xenobiotic metabolism *via* p-gp is tightly intertwined in a multi-dimensional network with the gut microbiota and the host innate immune system. But several questions arise from these results which remain to be answered, *e.g.*, What commensal community (if any) is responsible for driving murine colitis in the context of MDR1A deficiency? Does MDR1A deficiency sensitize otherwise tolerogenic mucosal immune cells to specific microbial ligands and/or xenobiotics? How do bioactive microbial metabolites modulate xenobiotic signaling *via* p-gp? Do xenobiotic compounds directly activate TLR (and other innate immune) signaling pathways to control p-gp activity? How is p-gp-mediated transport involved in microbial efflux from host cells?

GENE VARIANTS ASSOCIATED WITH IBD

Several studies have evaluated the potential association of human *ABCB1* gene polymorphisms with IBD susceptibility. The *ABCB1/MDR1* single nucleotide polymorphism C3435T, which has been correlated with lower expression of p-gp in the intestine[60], was found in patients with extensive UC in some populations[61-64], but not all[65-68]. Two meta-analyses[69,70] did not help to resolve this apparent contradiction, as they produced conflicting results as well. Using a robust gene-wide "block-free" haplotype tagging approach, Dr. Jack Satsangi's group previously identified six SNPs in the *ABCB1/MDR1* gene which were significantly associated with UC, but not CD, in their cohort[62]. In addition, a significant association of Ala893Ser/Thr (G2677) with IBD was shown in a large, multicentre North American study[71]. However, none of these *ABCB1/MDR1* gene variants were captured as major hits by the recent genome-wide screens in UC (personal communication, Dr. Judy Cho). These contrasting results may reflect differences in the populations studied. It is possible that certain *ABCB1/MDR1* gene associations may only be clearly detectable in refined case cohorts with distinct IBD sub-phenotypes.

So far, detailed studies examining the effects of these putative causal variants on gene function are missing in IBD. Based on the findings from the MDR1A KO colitis model, future studies will need to determine whether these variations in the *ABCB1/MDR1* gene may indeed alter xenobiotic metabolism, innate immune responses and host-commensal interactions in the human intestine. It must be clarified mechanistically how human *ABCB1/MDR1* gene defects may influence detoxification, antimicrobial defences and commensal composition.

RESISTANCE TO IBD THERAPY

Enhanced multidrug resistance *via* p-gp may limit the individual drug response. Several drugs central to IBD therapy represent p-gp substrates, including glucocorticoids[72] or cyclosporine[73]. Elevated p-gp expression levels have been shown in peripheral blood lymphocytes of those IBD patients who fail therapy with glucocorticoids[74]. High-dose administration of glucocorticoids may result in increased expression of *ABCB1/MDR1* mRNA in patients with UC[75]. Recently a human pathogenic Th17-cell subset which stably expresses p-gp was identified in patients with CD. These MDR1+-Th17 cells were refractory to different glucocorticosteroids[12], thus likely contributing to steroid-resistant chronic inflammation in IBD. Reversely, inhibition of p-gp significantly increases intracellular cortisol and cyclosporine levels *in vitro*, implying a potential target approach for overcoming the poor response to immunosuppressant therapy in refractory IBD[76]. However, *in vivo* proof remains so

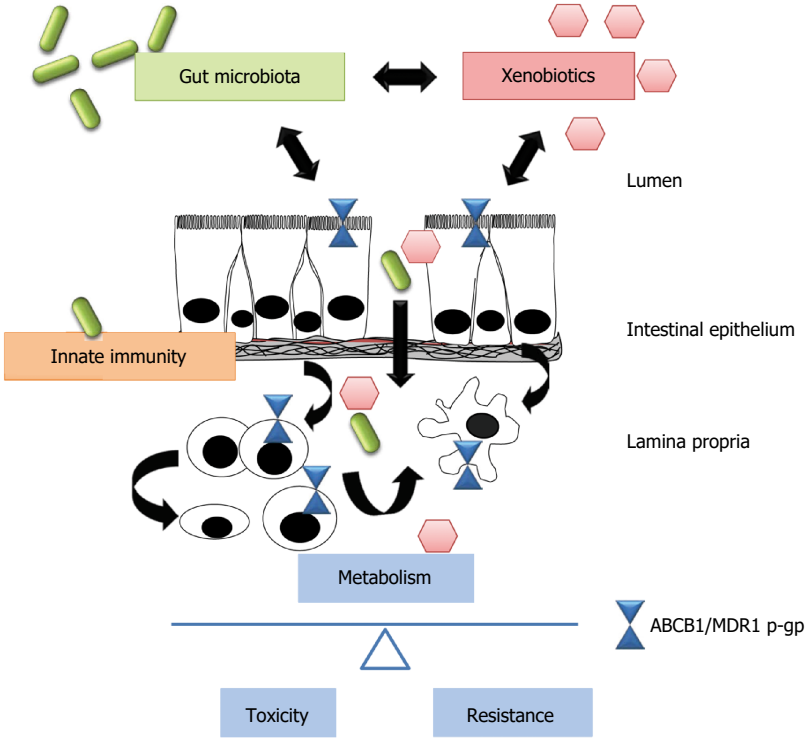

Figure 1 Xenobiotics, microbiota and host innate immunity interact in a multi-dimensional network in the gut. Disturbances of this equilibrium may alter xenobiotic metabolism *via ABCB1/MDR1* p-gp, favoring either drug toxicity or resistance in inflammatory bowel disease. However, these multiple interrelations remain to be further elucidated. P-gp: P-glycoprotein.

far lacking. Three generations of inhibitors of p-gp have largely failed to demonstrate any improvement in therapeutic efficacy in other clinical settings[77]. Future research will need to show whether the design of novel p-gp inhibitors, *e.g.*, based on recent advances in phytochemistry[78], would help overcome drug resistance in IBD.

CONCLUSION AND FUTURE PERSPECTIVE

It has become evident that gut microbiota and host innate immunity interact in a multi-dimensional network that controls xenobiotic metabolism to maintain normal mucosal homeostasis in the intestine. Imbalanced host-bacterial interactions may alter xenobiotic metabolism *via ABCB1/MDR1* p-gp, contributing to intestinal inflammatory processes, drug toxicity and resistance development in IBD (Figure 1).

Novel, large-scale approaches, including *in-silico* and complex computational tools[79], are now needed to provide in-depth elucidation of the possible pathways through which the gut microbiota may modify xenobiotics and vice versa as well as their combined metabolic effects *via ABCB1/MDR1* p-gp (and other transporters) on host immunity and functions in IBD pathogenesis.

The potential therapeutic value of *ABCB1/MDR1* p-gp as a molecular target requires further clarification in IBD. But it is clear that any p-gp targeting will become a delicate balancing act. Its ambivalent effects will make treatment development difficult. Future research will need to look at different therapeutic approaches, either to activate "underactive" p-gp in order to attenuate acute inflammation or to inactivate "overactive" p-gp in order to overcome therapy resistance.

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FRONTIER

Host pathogen interactions in *Helicobacter pylori* related gastric cancer

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Abstract

Helicobacter pylori (*H. pylori*), discovered in 1982, is a microaerophilic, spiral-shaped gram-negative bacterium that is able to colonize the human stomach. Nearly half of the world's population is infected by this pathogen. Its ability to induce gastritis, peptic ulcers, gastric cancer and mucosa-associated lymphoid tissue lymphoma has been confirmed. The susceptibility of an individual to these clinical outcomes is multifactorial and depends on *H. pylori* virulence, environmental factors, the genetic susceptibility of the host and the reactivity of the host immune system. Despite the host immune response, *H. pylori* infection can be difficult to eradicate. *H. pylori* is categorized as a group I carcinogen since this bacterium is responsible for the highest rate of cancer-related deaths worldwide. Early detection of cancer can be lifesaving. The 5-year survival rate for gastric cancer patients diagnosed in the early stages is nearly 90%. Gastric cancer is asymptomatic in the early stages but always progresses over time and begins to cause symptoms when untreated. In 97% of stomach cancer cases, cancer cells metastasize to other organs. *H. pylori* infection is responsible for nearly 60% of the intestinal-type gastric cancer cases but also influences the development of diffuse gastric cancer. The host genetic susceptibility depends on polymorphisms of genes involved in *H. pylori*-related inflammation and the cytokine response of gastric epithelial and immune cells. *H. pylori* strains differ in their ability to induce a deleterious inflammatory response. *H. pylori*-driven cytokines accelerate the inflammatory response and promote malignancy. Chronic *H. pylori* infection induces genetic instability in gastric epithelial cells and affects the DNA damage repair systems. Therefore, *H. pylori* infection should always be considered a pro-cancerous factor.

Key words: *Helicobacter pylori*; Host susceptibility; Carcinogenesis; Bacterial diversity

Core tip: In 1994 *Helicobacter pylori* (*H. pylori*) was classified by the International Agency for Research of Cancer as a class I human carcinogen for gastric cancer. Nearly 60% of the intestinal type gastric cancers are associated with *H. pylori* infections. Cancer risk rises if strain possess virulence factors: CagA, VacA and BabA. These bacteria promotes gastric carcinogenesis by increased DNA damage, impairment of repair processes, induction of mitochondrial DNA and genomic mutations. Nearly 98% of mucosa associated lymphoid tissue lymphomas are *H. pylori* dependent. We discuss correlation between *H. pylori* and gastric cancer in the light of bacterial and host genetic variability.

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BIOGRAPHY

With a master degree on biology, microbiology as specialty, upon her PhD on Immunology in 1991, Magdalena Chmiela (Figure 1) was nominated in 2005 on the position of permanent Professor (medical microbiology, immunology) at the Faculty of Biology and Environmental Protection, University of Lodz, Poland. She is currently head of the Department of Immunology and Infectious Biology at the Institute of Microbiology, Biotechnology and Immunology. For more than 30 years her research concerns the immunology of infectious diseases including: immune processes regulating host-pathogen interactions, bacterial virulence factors that determine the course of infections, the use of microorganisms in the design and manufacture of biological components for potential therapeutic use, prevention and diagnostic. With particular attention she leads research on *Helicobacter pylori* (*H. pylori*) infections, which are responsible for gastric and duodenal ulcers and even stomach cancers. Work on this subject she began in 1992, being a member of the research team at the Department of Medical Microbiology Lund University in Sweden. She also conducts research about Campylobacter sp. With her experience she published numerous papers, review articles, coordinated and participated in a number of research projects and evaluated them as an expert. She is a member of the Scientific Council of the Institute of Medical Biology, Polish Academy of Sciences; editorial board member of the *World Journal of Gastroenterol* (2014-2017); member of American Society for Microbiology and Polish Society for Microbiology. She shares her professional activity between research work and academic professor activity.

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INTRODUCTION

The stomach is considered a hostile environment for microorganisms. The acidic pH and peristaltic movements of the stomach prevent colonization by pathogens. In 1982, Barry Marshall and Robin Warren revolutionized the concept of gastroduodenal diseases by the discovery of *H. pylori* and by proving that these gram-negative bacteria cause infections in humans due to colonization of the stomach. If the pathogen is not eradicated by the immune system of the host, it stimulates the development of chronic inflammation. The pathogen is a major agent in gastritis and peptic ulcers (PU), which were previously thought to be caused by stress and diet. Now it is known that *H. pylori* is also involved in the development of gastric cancer (GC).

The aim of this review is to present a brief overview of how *H. pylori* infection impacts tumorigenesis. Gastric adenocarcinoma has the second highest mortality rate in the world. Nearly half of the world's population is infected by *H. pylori.* Various structural components and soluble factors of *H. pylori* enable these microbes to colonize the stomach and induce an inflammatory response. Close contact with an infected person facilitates transmission of the pathogen by an oral-oral or oral-fecal route. Clinical outcomes that are linked with *H. pylori* infection include chronic inflammation of the gastric mucosa, gastric and duodenal ulcers (DUs) and GC. Although a correlation between the pathogen and carcinogenesis has been established, more studies are needed to understand specific mechanisms, the diversity of infectious agents, and the genetic susceptibility and immune profile of the host.

MICROBIOLOGICAL ASPECTS OF *H. PYLORI*

Primary bacteriological features

H. pylori is considered the most prevalent human pathogen, and its evolution appears to have been very effective since the bacterium has developed several strategies to cause infection[1]. *H. pylori* had escaped the attention of researchers until Barry Marshall and Robin Warren published data on the curved bacterium that colonizes the human stomach[2]. Substantial alterations have been made concerning the disease causation after intensive studies on *H. pylori*[3]. This pathogenic microorganism was first named *Campylobacter pyloridis*. It was only after facing important genotypic and phenotypic dissimilarities with other bacteria in the *Campylobacter* genus that a decision was made to create a new genus: *Helicobacter.* It is now commonly accepted that this gram-negative, microaerophilic, flagellated microorganism induces chronic active gastritis (asymptomatic or symptomatic), peptic ulcer disease and duodenal ulcers in humans; it is also related to GC[4,5].

Virulence factors

The colonization of epithelial cells of the stomach by *H. pylori* begins with the binding of these bacteria with epithelial cell receptors. Then the bacteria escape of host defense mechanisms, induce inflammatory responses, which allow acquisition of nutrients for successful replication[6]. Majour *H. pylori* adhesins belong to the family of proteins localized in outer membrain of bacterial cells. The blood group antigen-binding adhesin A (BabA) and sialic acid binding adhesin (SabA) are the most important adhesisns of *H. pylori*[7-11]. Also other OMPs, such as HopZ and OipA play a role of adhesins. It has been shown that OipA induces more intensive inflammatory response due to neutrophil infiltration and promotes the development of duodenal ulcer and gastric cancer[7]. Urease elevates the acidic pH of the stomach and unipolar flagella facilitate penetration of mucus[3]. The ability to glycosylate host cholesterol is crucial for the virulence and antibiotic resistance of *H. pylori*[12]. *H. pylori* lipopolysaccharide (LPS), due to its structural features, induces a poor immune response and helps the bacteria develop into a chronic infection[13-18]. *H. pylori* LPS may carry various human Lewis (Le)-like antigens, which may play a role in autoimmunity. Specifically, Le^x determinants in O antigen of *H. pylori* LPS may facilitate the adherence of bacterial cells to gastric epithelium. This process involves the binding of gastric receptor β-galactoside-binding lectin (galectin-3)[19-21]. The *H. pylori* outer membrane vesicles are an alternative vehicle for the distribution of bacterial virulence factors and antigens[22,23]. The major virulence factors of *H. pylori* are encoded by genes within the pathogenicity island (PAI). The cytotoxin-associated gene A (CagA) protein is one of the most important *H. pylori* virulence factors. CagA is encoded by the *cagA* gene and translocated to the host gastric epithelial cells through a type Ⅳ secretion system[24-28]. A correlation between the presence of CagA in *H. pylori* strains and more severe inflammatory responses and a higher risk of gastric cancer has been shown[26-29]. Other virulence proteins include vacuolating cytotoxin A (VacA), BabA and SabA[9,10,30,31]. VacA induces vacuolation of gastric epithelial cells as well as cell apoptosis and disrupts the gastric epithelial barrier function[28]. BabA and SabA are adhesins, and SabA is essential for nonopsonic activation of human neutrophils[9,7]. BabA interacts with the Le^b blood group antigen on epithelial cells, and the *babA2* gene is associated with DU and GC[10]. SabA is known to bind sialyl-dimeric-Le^x[8], as well as sialylated Le^a[9]. Malignant transformation is linked with pronounced expression of Le^a, sialylated Le^a and sialyl-dimeric-Le^x, however, knowledge about the role of SabA in tumorigenesis is still limited[9].

Immune system evasion strategies

Blaser (1993) proposed a model in which both the host and the parasite adapt to downregulate the inflammatory response to promote survival and to continue colonization of the niche[32-34]. Pathogen-associated molecular patterns (PAMPs) are various molecules of pathogenic microorganisms that in normal conditions are recognized by pattern recognition receptors (PRRs) resulting in triggering of the inflammatory response. *H. pylori* possess several mechanisms that prevent their recognition *via* Toll-like receptors (TLRs): (1) changing and rearranging LPS and flagellin; and (2) molecular mimicry between human Lewis and ABO blood group antigens and bacterial compounds, which confuses immune cells and prevents recognition of the pathogen[21,35,36]. It has been shown that the *H. pylori* flagellin is not detected by specific PRRs, and it does not stimulate the production of interleukin (IL)-8. As a result, chemotaxis of immune cells to the site of infection and phagocytosis of *H. pylori* are diminished[37].

Prevention of phagocytic killing has been demonstrated to be more efficient due to delayed polymerization of actin and inhibition of phagosome and phagolysosome formation[28,38]. The primary host immune response mechanisms, such as phagocytosis and natural killer (NK) cell activity, have been found to be downregulated by *H. pylori* LPS[17,18,39,40]. Adaptive immunity is also targeted by *H. pylori* compounds[1,15,41,42]. They affect antigen presentation by inducing macrophage apoptosis and by diminishing dendritic cell (DC) and macrophage maturation[18,43]. The expression of programmed death 1 ligand-1 (B7-H1 integrin) on gastric epithelial cells modulates T cell trafficking during *H. pylori* infection. The function of B7-H1 is to inhibit effector T lymphocytes and stimulate DCs to increase secretion of the anti-inflammatory cytokine IL-10. B7-H1, by join-

ing programmed cell death receptor 1 on the surface of T cells, inhibits proliferation and differentiation of naïve T lymphocytes and promotes the activity of regulatory cells, which downregulates effector T lymphocytes. Regulatory T cells, which possess the ability to suppress anti-tumor and anti-infectious responses are identified on the basis of cluster differentiation (CD) markers and forkhead box P3 (FOXP3) as CD4(+)CD25(high) and FOXP3-positive. Enarsson *et al*[44] studied regulatory T lymphocytes in stomach tissue in *H. pylori* positive patients in terms of their activity and the expression of homing receptors. The increased number of regulatory T cells has been detected in gastric tissue of patients with gastric tumor *vs* non-tumor patients. Regulatory T lymphocytes suppressed *H. pylori*-induced T cell proliferation and interferon (IFN)-γ production. Furthermore, these regulatory T lymphocytes expressed increased levels of l-selectin and C-C chemokine receptor 4, than the cells lacking regulatory function. These receptors may be involved in the infiltration of regulatory lymphocytes specific to *H. pylori* antigens present in gastric tissue in *H. pylori* infected individuals. However, low activity of T regulatory cells may promote the maintenance of the infection and potentially the propagation of tumor cells[45]. The suppression of the activity of memory T lymphocytes, which enables a chronic infection, has been confirmed by other study groups[45-48]. The role of regulatory T lymphocytes can be related to the inhibition of the inflammatory response driven by IL-17 delivered by T helper (Th) 17 lymphocytes[49-52].

Different studies have shown that humoral response against *H. pylori* is less essential in the defense against this pathogen.The study on mice lacking B lymphocytes showed that gastritis, which developed in animals immunized with prophylactic vaccine was not related to B-cells. The response was similar to that of non immunized mice[53,54]. It can be concluded that antibody responses may not promote protection. However, a correlation between high levels of serum anti-*H. pylori* IgG and IgA and the development of gastritis, duodenal ulcers and gastric cancer has been shown[1].

PATHOGENIC ACTIVITY OF *H. PYLORI* IN THE HOST ORGANISM

Epidemiology

There is an inverse association between socioeconomic status and the rate of infection[54]. Analyses have been conducted to test whether animals or water can be sources of *H. pylori* infection. Only a few of the animal case studies showed positive results, leading to the conclusion that the infection cycle might include humans, the environment and animals. However, the water case studies failed to support the hypothesis that water is an environmental reservoir of *H. pylori*[55]. The principal method of spreading *H. pylori* infection is intrapersonal transmission. This has been confirmed by the high percentage of infections that are spread between close relatives, especially between a mother and her children[56].

Clinical complications

The clinical aspects of *H. pylori* infection vary from gastritis and peptic ulcers to gastric cancer. It has been suggested that the pathogen might also be associated with several extragastric diseases. Shortly after initial infection of the host, acute gastritis develops that is related to hypochlorhydria and to the loss of acid secretion. Acute gastritis does not last long, but in the majority of subjects, the immune response is unable to eradicate the infection, and as a consequence, chronic gastritis is induced. According to various studies, half of the world's population may suffer from chronic gastritis, which can be manifested in one of three forms: (1) antral-predominant; (2) corpus-predominant; and (3) diffuse. These pathologies lead to different consequences, which they favorably induce. Specifically, antral-predominant gastritis promotes duodenal ulcers whereas corpus-predominant gastritis promotes gastric ulcers, which may lead to metaplasia and adenocarcinoma; and diffuse gastritis is related to reduced acid secretion in the stomach[57-59]. In general *H. pylori* infections are responsible for 95% of duodenal ulcer cases and 85% of gastric ulcers. Nonsteroidal anti-inflammatory drugs are responsible for the cases that are not related to pathogen-induced inflammation[3]. Extragastric diseases potentially related to *H. pylori* include idiopathic thrombocytopenic purpura and iron deficiency anemia[60-66]. The influence of pathogen-induced inflammation has also been considered in several dermatological disorders, diabetes and cardiovascular, and pulmonary disease[67-76]. The connection between *H. pylori*-induced inflammation and cardiovascular disease was reported in 1994 by Mendall *et al*[77], and this work was then followed by many other studies[78-86]. However, the association between *H. pylori* infection and extragastric disease remains unclear. Therefore, the recommendation for *H. pylori* treatment is irrelevant[3]. According to recent data, *H. pylori* infection might facilitate the onset of hepatic encephalopathy[87]. The theory of *H. pylori* influence in diabetes is very recent. Specifically, $CagA^+$ strains are thought to enhance the risk of diabetic complications[88-92]. There is no doubt about the beneficial effect of the infection against endoscopic gastroesophageal reflux disease[93-95]. However, *H. pylori* infection may potentially prevent the development of adenocarcinoma of esophagus[96]. Based on a case-control study, infection with *H. pylori*, particularly the $CagA^+$ strain, has been found to be inversely associated with Barrett's esophagus[97]. *H. pylori* infection likely has a beneficial role in maturation of the immune system in the early stages of life

and prevents asthma development in the future[98-103]. The most dangerous clinical aspects of *H. pylori* are gastric cancer[29,48,104-108] and mucosa-associated lymphoid tissue (MALT) lymphoma[109-111]. The role of *H. pylori* in destruction of epithelial cell nuclei and mitochondrial DNA has been confirmed. This mutagenic effect is in part related to downregulation of the expression, as well as the activity, of DNA repair pathways. Machado *et al*[112] demonstrated that infection of gastric adenocarcinoma cells with *H. pylori* induced mutations in mitochondrial DNA and decreased the DNA content. The increased frequency of mutations in mitochondrial DNA was related to diminished effectiveness of DNA repair mechanisms. They showed that apurinic/apyrimidinic (AP) endonuclease-1 and Y-box-binding protein 1 mitochondrial base excision repair and mismatch repair systems are involved in DNA repair during *H. pylori* infection[112].

ROLE OF *H. PYLORI* IN TUMORIGENESIS

From carcinogenesis to gastric cancer

Accumulation of numerous mutations in DNA of gastric epithelial cells, resulting in activation of oncogens or inactivation of tumor suppressor genes promotes the development of gastric cancer[113,114].

Nearly 120 years ago, the first gastrectomy was performed to treat gastric cancer. Since then, tumor resection in the stomach has been the standard method of treatment. On average, only 15%-20% of patients live up to 5 years after resection. Patients diagnosed in the early stages of gastric cancer have a 5-year survival of nearly 90%[115,116]. Cancer in early stages can be surgically curable because of its local development. The advancement of gastric cancer is directly proportional to the involvement of regional and non-regional lymphoid nodes, as well as organ metastasis. If the cancer is scattered throughout the body, surgical methods that treat local cancer are not effective. In these cases, implementation of additional cytostatic and hormonal treatment is necessary. Approximately 97% of gastric cancer cases are linked with metastasis. Sarcomas and non-Hodgkin's lymphoma rarely occur. Every year, 670000 new cancer cases are registered around the world. Gastric cancer is two-times more frequent in men than in women. It usually occurs between the ages of 50 and 70, but lately, it is increasingly being detected in young people. Gastric cancer grows by contiguous extension (direct infiltration) to other organs, such as the pancreas, liver, transverse colon, duodenum and esophagus, as well as through the peritoneum to the recto-uterine Douglas pouch. Metastatic cancer spreads through the ovaries and lymphatic or blood vessels[115-117].

In 1965, Lauren described two histologically different stomach adenocarcinomas - diffuse and intestinal[118]. The diffuse type is considered an endemic cancer type. Diffuse adenocarcinoma affects mostly women and younger populations. The typical development area of the endemic type is the proximal portion of the stomach. It often coexists with the A blood group, which suggests a possible genetic basis for tumor formation. The intestinal type is related to preneoplastic changes, such as chronic atrophic gastritis and intestinal metaplasia of mucous membranes. This type concerns tumors in the peripheral part of the stomach. Intestinal adenocarcinoma is an epidemic type of cancer because it occurs in regions with a high risk of gastric cancer morbidity. It affects mostly men and older populations[116,118].

Gastric cancer as a consequence of H. pylori infection

The discovery of *H. pylori* confirmed that the etiology of chronic gastritis and the "precancerous cascade" resulting in cancer formation is associated with *H. pylori* infection[119]. Now, it is commonly accepted that *H. pylori* is a gastric cancer carcinogen since in 1994, *H. pylori* has been included by the International Agency for Research on Cancer to class I carcinogens[120]. Nearly 60% of intestinal-type gastric cancers are associated with such infections[121,122]. Over years, patients develop acute and then atrophic gastritis, followed by intestinal metaplasia, dysplasia and carcinoma. *H. pylori* infection also stimulates the development of diffuse type adenocarcinoma by causing pangastritis and rugal hyperplastic gastritis[123]. Cancer risk rises if virulence factors, such as CagA, VacA and BabA, are present in the *H. pylori* strain[28,29,124]. However, infection with *H. pylori* CagA$^+$ strains may potentially diminish the risk of adenocarcinoma of esophagus and gastric cardia[125]. There is an increasing interest on the role *H. pylori oipA* positive strains in the pathogenesis of gastric ulcer and cancer. When *oipA* is present, the functional "on" status of this gene was associated with increased risk of these diseases compared with gastritis and functional dyspepsia controls[7].

Environmental factors also stimulate the initiation of atrophic changes and decrease the secretion of hydrochloric acid. Elevated pH of the gastric juice facilitates bacterial colonization, causing further damage to epithelial cells. In addition, nitrates in foods are precursors of nitrosamines, which cause intestinal metaplasia and dysplasia (abnormal epithelial differentiation, in the form of improper development of the cells with the loss of ability to differentiate)[126,127].

Machado *et al*[128] have proposed three possible mechanisms of initiation of gastric cancer in response to *H. pylori* infection: damage of epithelial cell DNA combined with downregulation of repair processes, mitochondrial DNA mutations, and appearance of transient mutator phenotype. Park *et al*[129] showed that after eradication of *H. pylori* the expression of proteins consisting DNA mismatch repair (MMR) system was increased. This proved that gastric inflammation due

to *H. pylori* infection impairs MMR[129]. Kim *et al*[130] co-cultured gastric cell lines with *H. pylori* and the proteins (MutS and MutL) of DNA MMR, and examined quantitatively RNA levels. RNA of both proteins was reduced after exposure to *H. pylori*. Kidane *et al*[131] showed that damage of epithelial cell DNA due to oxidative stress, which increases during *H. pylori* infection is under control of base excision repair system and its effectiveness can be crucial for preventing genomic stability in response to *H. pylori* induced disorders. Toller *et al*[132] showed that *H. pylori* strains having the BabA adhesin are very effective in inducing double-strand breaks.

Biomarkers for detection of gastric cancer

Early detection of adenocarcinoma is essential. The 5-year survival rate for patients suffering from advanced stomach cancer is lower than 30%. Currently, endoscopic surveillance is the most applicable method for cancer detection. However, endoscopy has disadvantages, such as the invasiveness of the test and its high cost. It has been shown that appropriate biomarkers provide information about the diagnosis, prognosis and recurrence of cancer, as well as the optimal therapy[133]. Nevertheless, gastric cancer biomarkers such as pepsinogen, gastrin or *H. pylori* serology combined with pepsinogen (PG), do not indicate very precisely the state of the patient[134]. Pepsinogen is produced in the stomach as pepsinogen I (PGI) and pepsinogen II (PGII). The blood levels of PGI and PGI/PGII change during atrophic gastritis due to destruction of gastric glands. A research study involving approximately 300000 participants was performed in order to verify this observation. The results showed that out of 600 patients with atrophic gastritis, one developed stomach cancer. A PGI/PGII ratio within the normal range was very accurate negative predictor of an unhealthy stomach[135,136]. Gastrin is also considered a biomarker for gastric atrophy, but the connection between the biomarker and the disease is complex[137,138]. Gastrin is produced in the antrum of the stomach. In the case of antrum atrophic gastritis, the biomarker indicates a low gastrin level, but in the case of corpus atrophic gastritis, the gastrin level is increased. Generally, low and high levels of gastrin predict atrophic gastritis and gastric cancer, respectively. However, gastrin as a biomarker does not provide information about the cancer stage. Furthermore, combined tests for the detection of *H. pylori* and the PGI/PGII value also help to detect gastric cancer[139]. Patients with a seronegative *H. pylori* result and PG within the norm have very low rates of cancer susceptibility. The risk rises in cases of *H. pylori* seropositivity and low PGI/PGII values, suggesting the presence of gastric atrophy. However, negative *H. pylori* testing accompanied by low PGI/PGII indicates the manifestation of autoimmune metaplastic atrophic gastritis. This condition is linked with advanced grades of metaplasia in the stomach[54,134].

A novel group of biomarkers is microRNAs (miRNAs), which are nucleotides that modulate the expression of genes. miRNAs influence cell proliferation and differentiation and may act as oncogenes. Cancer-related miRNAs have been found in the blood stream and can be detected noninvasively. Levels of miRNAs in healthy patients provide information about cancer susceptibility. However, in patients with gastric cancer, the levels of the biomarkers are associated with cancer stage, metastasis, recurrence and resistance to treatment. The inconsistent outcomes from several studies on miRNAs note the necessity for more tests on this biomarker[133,134].

Other cancers potentially related to H. pylori

H. pylori infection is linked to MALT lymphoma[109-111]. Nearly 98% of MALT lymphomas are *H. pylori* dependent because prolonged infection with the pathogen leads to proliferation of the lymphoid tissue. Eradication of *H. pylori* infection used as a cure for *H. pylori*-positive MALT lymphoma was found to correlate with the remission in 60%-80% of MALT-lymphoma cases[111,140]. The presence of *H. pylori* in the host elevates the risk of developing other lymphomas, such as diffuse large B cell lymphoma and ocular adnexal lymphoma[3]. Contradictory results leave unclear the influence of the pathogen and of eradication therapy on carcinogenesis. Several studies have shown that *H. pylori* infection is correlated with laryngeal squamous cell carcinoma[140-142]. CagA-positive strains were found to cause a more severe condition and reduce the survival rate. However, not all cases confirm such an association[3]. Colorectal cancer development is also considered to be related to *H. pylori* infection[143-146]. High rates of mortality in specific regions from colorectal and stomach cancer, as well as high prevalence of the pathogen in critical colorectal adenomas point to *H. pylori* as a mutual risk factor. Some studies are in opposition to this theory because the pathomechanisms are not fully understood. An association between *H. pylori* infection and hepatocellular carcinoma has been suggested[147,148]. Esmat *et al*[149] have suggested that the presence of CagA positive *H. pylori* strains in the liver may cause progression of hepatocellular carcinoma due to infection with hepatitis C virus (HCV). The link between *H. pylori* infection and hepatic carcinoma has been confirmed by detection of genetic material of these bacteria in hepatic tissue[150]. The possibility of corelation between *H. pylori* infections and the development of pancreatic cancer has been suggested[151]. The role of gastric carriage of *H. pylori* $CagA^+$ strains, in increasing a risk for gastric ulcer as well as gastric and pancreatic cancers was shown on the basis of seroprevalence of *H. pylori* by Stolzenberg-Solomon

et al[152]. Meta-analysis performed by Trikudanathan *et al*[153], suggested a reduced statistically significant association. In addition, other data support the hypothesis of a correlation between pancreatic cancer and *H. pylori* as well as the ABO genotype due to its role in gastric secretion and the secretory activity of the pancreas[154-156].

H. PYLORI DIVERSITY *VS* GASTRIC CANCER RISK

CagA variation

The course of *H. pylori* infection depends on complex interactions between the microbial agent and the host genetic background, as well as host immune profile. *H. pylori* is a diverse microorganism. Specific features of an individual strain can determine the severity of inflammation and its consequences, including the promotion of malignancy. This diversity refers to the most important virulence factors, such as CagA, VacA toxin and OMPs.

CagA induces *in vitro*, the 'hummingbird' phenotype of epithelial cells of the stomach with symptoms of cell elongation. These cellular changes are similar to epithelial-mesenchymal transition (EMT), which occurs during development of gastric cancer stem cells (CSC). *H. pylori* CagA promotes EMT phenotype, which was studied on the basis of both mesenchymal markers and CD 44 molecules associated with CSC[157]. The presence of CagA with phosphorylated Glu-Pro-Ile-Ala-Tyr, called the EPIYA motif, in host cells induces changes in the cytoskeleton, modifications of intercellular connections and deregulation of the expression of genes encoding transcription factors. EPIYA motifs in the C terminal region of CagA determine its interaction with numerous host proteins. Multimeric, non-phosphorylated CagA protein enhances the activity of phosphorylated CagA protein and contributes to the loss of cell polarity[11,28]. Within the EPIYA motif there is a phosphate acceptor tyrosine domain. This region is polymorphic since it contains different numbers of EPIYA motifs. Moreover, the diversity was also found in regions among EPIYA sequences. The length polymorphism at the 3' end of the *cagA* gene results with increased phosphorylation of CagA protein, which enhance its biological activity and promotes more severe disease outcome[158]. Four EPIYA motifs have been described: -A, -B, -C, and -D. Their combination depends of geographic regions[159]. In general Western *H. pylori* strains possess EPIYA -A, -B, and -C whereas strains from East Asian region EPIYA -A, -B, and -D. The East Asian CagA-positive *H. pylori* strains are more closely associated with gastric cancer[160].

Vaziri *et al*[161] studied the influence of EPIYA motifs on the transcriptions of genes related to gastric cancer by using transfected gastric cancer AGS cell line with a eucaryotic vector carrying the *cagA* gene: ABC and ABCCC types. They found that the CagA oncoprotein of ABCCC type can induce intestinal metaplasia, IL-8 production by epithelial cells, dysfunction of Crk adaptor proteins, and anti-apoptotic and carcinogenic effects more intensively than the CagA protein of the ABC type.

The association between the number of EPIYA-C regions and increased CagA tyrosine phosphorylation, protein tyrosine phosphatase (SHP)-2 binding activity, cytoskeletal alterations, IL-8 expression in gastric mucosa, development of the hummingbird cell phenotype and severe disease frequency was found[162].

Western and East Asian CagA proteins differ in sequence among the EPIYA motifs. The FPLKRHD-KVDDLSKV sequence, which is present in Western type CagA in East-Asian type CagA is substituted by KIASAGKGVGGFSGA sequence. This amino acid sequence variation is supposed to be responsible for the higher frequency of gastric cancer in Japan as compared to the Western coutries[162]. Jones *et al*[159] verified that the East Asian EPIYA phenotype is closely related with disease development. Phosphorylated CagA regions are primarily EPIYA-C and -D sites, which are required for binding to SHP-2 and its activation[159].

Chattopadhyay *et al*[158] have suggested that in India, the infections related to different structures of CagA can be multiple. In this case the disease course is not determined by a particular type of CagA. They concluded that the risk of developing the disease is also associated with polymorphism of genes encoding other *H. pylori* proteins, as well as with the host genotype[158].

Research on a group of 436 Brazilian patients by Batista *et al*[163] showed that *H. pylori* strains in this region are the Western type and that there is a tight correlation between the number of EPIYA-C segments and increased risk of gastric carcinoma but not duodenal ulcers[163] similarly as in Caucasian population from Italy and American patients in Texas[164,165].

Regardless of the C/D type, most CagA molecules include single A- and B- tyrosine phosphorylation motifs (TPMs) that do not undergo simultaneous tyrosine phosphorylation[166]. Phosphorylated A- or B-TPMs have host interaction partners distinct from C- or D-TPMs and from each other, suggesting unique signaling functions. Zhang *et al*[166] showed that in the Western population, also, the polymorphism of the EPIYA-B motifs influences the frequency of disease development, suggesting that a single nucleotide polymorphism in a major bacterial interactive compound could promote a disease outcome. In this study, the CagA B-TPM sequences showed the highest variability. The EPIYA motif was present in 72.6% of B-TPMs. However, other EPIYA-like motifs have been identified (EPIYT, ESIYT, ESIYA, GSIYD). The analysis carried out by Zhang *et al*[166] demonstrated that the association of EPIYT segments with gastric cancer is lower than the EPIYA motifs.

The correlation, which was found between EPIYA

motifs and the level of IL-8 as well as a strength of inflammatory response in gastic mucosa may depend on the geographical region[167]. Fajardo *et al*[162] and Reyes-Leon *et al*[167] did not show correlation between the number of EPIYA-C motifs and IL-8 induction in the Columbian as well as Mexican population whereas Argent *et al*[168] obtained an opposite results for English population. Interestingly, Mexican and Columbian *H. pylori* strains share common predominant polymorphisms (ABC and ABCC). Hatakeyama has suggested that CagA is involved in gastric carcinogenic processes through a hit-and-run mechanism, in which pro-oncogenic activities of CagA are successively taken over by a series of genetic and/or epigenetic alterations compiled in cancer-predisposing cells during long-lasting infection with *cagA*$^+$ *H. pylori*[29].

VacA variants

VacA is a polymorphic toxin with pore forming activity and there are different allels of *vacA* gene within *H. pylori* strains. VacA is composed of four regions, which are further subdivided. The signal (s) region, which includes the N-terminus and a signal sequence is classified as s1 or s2[169]. The s region influences the formation of anion channel[170]. The mid (m) region, which affects host cell tropism, is classified as m1 or m2[169,170]. The intermediate (i) region is classified as i1, i2, or i3[169]. This region determines the vacuolating and cancerogenic activity of VacA toxin[171]. The d region means the deletion of 81 bp between the i- and m-regions. Without deletion it is classified as d1 or d2 if a 69 to 89 base pair deletion is present[169,171]. VacA virulence depends on the combination of individual parts. The *vacA* s1/m1 alleles determine high cytotoxic activity of VacA. By comparison the s1/m2 and s2/m2 genotypes are not cytotoxic. The s1/m1 profile is strongly correlated with the outcome of duodenal ulcers, peptic ulcer disease, progression of preneoplastic lesions, and gastric cancer[169,170]. Ogiwara *et al*[172] showed that the risk of gastric cancer in Western countries is related to the s1, m1, i1, and d1 polymorphisms, which are potentially linked with an increased neutrophil infiltration and gastric mucosal atrophy[171]. However, in other studies such a correlation was not found in East Asian countries[171,172].

It was found that i1 variants of the VacA protein have stronger vacuolating activity than i2 variants. Moreover, the i1 region is considered a better predictor of disease severity than the s1 and m1 variants in Western strains. The i region may contain A, B, and C polymorphic domains. The VacA toxicity depends on B and C part[170].

OMPs

Genes encoding OMPs consist 4% of *H. pylori* genome. Many *H. pylori* OMPs belong to OMP family 1, which contains various *H. pylori* outer membrane proteins (Hop) and Hop-related proteins (Hor). *H. pylori* OMPs are crucial for adaptation of the pathogen to the host. They play a role in bacterial movement and adhesion to gastric tissue[7]. Adhesins with known binding specificity include BabA (HopS), which binds Lewis^b, a fucosylated blood-group antigen that is present in gastric tissue[31] and SabA (HopP), which is a sialic acid-binding adhesin associated with higher colonization density in humans[173]. The *alpAB* locus has been shown to encode the outer membrane adhesins AlpA and AlpB, which bind laminin[174]. The HorB protein is another adhesin, however, its ligand has not been identified[175].

The best-characterized OMP of *H. pylori* is BabA, which is encoded by the *babA2* gene[176]. Research carried out by Torres *et al*[176] on a group of 130 *H. pylori* isolates from dyspeptic Cuban patients showed that the presence of a 'triple positive' genotype (*vacAs1*, *cagA* and *babA2*) (56.2% isolates) is correlated with the appearance of peptic ulcers, intestinal metaplasia and gastric cancer. Infection with these strains was found to be associated with a higher degree of inflammation and gastroduodenal lesions[176].

Research on 167 *H. pylori*-positive patients conducted by Zambon *et al*[177] allowed patients to be divided to four groups (A, B, C and D) on the basis of bacterial genotypes: *cagA*(-), *s2 m2*, *babA2*(-); *cagA*(+), *s1 m1*, *babA2*(+); *cagA*(+), *s1 m2*, *babA2*(+); *cagA*(+), *s1 m2*, *babA2*(-), respectively, that differ in their ability to induce gastrointestinal diseases. *H. pylori* strains of group B induced the worst inflammatory response including intestinal metaplasia[177]. Moreover, a relationship between *cagA* and the *s1* and *m1* alleles of *vacA* and *oipA* was found. By comparison, *H. pylori* strains without *cagA* were usually *babA2*(-) and *oipA*(-) and they held the *s2* and *m2* *vacA* alleles. This observation confirmed the role of the pathogenicity island as the main vehicle of virulence genes[177].

Another important Hop is HopH, encoded by the *HP0638/hopH* gene[178]. The *hopH* genotype has been foud related to *H. pylori* virulence markers including *vacAs1, vacAm1, babA2,* with the strongest association to *cagA.* The association of the *HopH* gene with gastric disorders could be due to promotion of increased bacterial adherence and colonization by the HopH. The expression of *hopH* has been found regulated by phase variation within a CT dinucleotide repeat motif[178].

Host genetic susceptibility and immune profile

The long lasting inflammation induced by *H. pylori* infection is followed by DNA damage, the impairement of repair processes and increased rate of mutations. These phenomena promote the development of *H. pylori*-related gastric carcinogenesis[128-132,179].

Pattern recognition receptors

Pathogens possess many conservative PAMPs. These structures, which are present in various groups of

microorganisms, have not changed during evolution and do not occur in human organisms.These compounds are recognized by PRRs, which are deposited on immune cells as well as epithelial cells and vascular endothelium. TLRs and damage-associated molecular patterns (DAMPs) are representative PRRs[180,181].

Various groups of receptors are simultaneously engaged in recognition of *H. pylori* compounds and the development of gastric cancer. These are TLR2, TLR3, TLR4, TLR5, and TLR9; nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), such as NOD1, NOD2, and NLRP3 (NLR family pyrin domain containing 3); dendritic cell-specific intercellular grabbing non-integrin; retinoic acid-inducible gene (RIG)-I-like receptors (RIG-I); and melanoma differentiation associated protein 5. Polymorphisms in genes, which are involved in the signaling cascades *via* TLR, NLR, apoptosis-associated speck-like protein, and caspase recruitment domain containing protein 8 (CARD8) can increase the risk of *H. pylori* infection and gastric cancer[182,183]. This can happen because the dysfunction of genes, which are involved in cell signaling pathways *via* the above receptors may significantly modulate the host immune response during *H. pylori* infection[183].

TLR4

TLRs recognize various *H. pylori* PAMPs, including flagellin (TLR5) and unmethylated CpG motifs (TLR9) as well as LPS (TLR4/TLR2)[183].

The expression of TLR2, TLR4 and TLR5 increases during gastric dysplasia and especially a strong correlation between TLR4 and gastric carcinoma has been suggested[184]. Additionally, Chochi *et al*[185] found that binding of *H. pylori* LPS to TLR4 resulted in increased growth of gastric adenocarcinoma. On this way also antitumor activity of human mononuclear cells was diminished.

In recent studies, much attention has been paid to the influence of TLR receptor polymorphisms on the development of diseases associated with *H. pylori* infection. Single nucleotide polymorphisms (SNPs) of the TLR4 receptor were connected with an increased risk of gastric carcinoma, including *TLR4* rs4986790 (Asp299Gly)[186,187], *TLR4* rs4986791 (Thr399Ile)[187], *TLR4* rs10116253, *TLR4* rs10983755, *TLR4* rs11536889 (C3725G/C)[182], *TLR4* rs1927911[183]. *TLR4* Asp299Gly and Thr399Ile polymorphisms located in the encoding region have been considered the most important since they diminish the stability of the TLR4 extracellular domain[182,187].

Another study conducted by Bagheri *et al*[186] on a group of 195 patients with *H. pylori* infection and 241 *H. pylori* not-infected individuals confirmed that the increased frequency of *TLR4* (Asp299Gly) G and DG alleles was related to chronic active gastritis. An A-G substitution at 896 bp was associated with a decreased response to LPS *in vivo* and *in vitro* and an increased risk of inflammatory disease. The results obtained by Castaño-Rodríguez *et al*[182] confirmed that in the Western population the *TLR4* Asp299Gly G allele as well as the *TLR4* rs11536889 C allele and the CC genotype increased the risk of gastric cancer or other inflammation-related cancers. These results indicate that there is a relationship between the *TLR4* rs11536889 polymorphism and increased incidence of cancer, which is consistent with the fact that the *TLR4* rs11536889 polymorphism is located in the center of the 2818-bp *TLR4* 3'UTR and, therefore, may affect mRNA stability. However, other studies of polymorphism investigated in Asian and Caucasian individuals have shown different risk associations with gastric cancer in an ethnic-specific manner[182].

TLR2

In *H. pylori* infection, much attention is also focused on TLR2. It has been shown[188] that *H. pylori* LPS as TLR2 ligand induces the secretion of chemokines by gastric epithelial cells due to acting on tribbles 3 (TRIB3) protein, which is involved in the expression of the nuclear factor NF-κB. However, both TLR4 and TLR2 are engaged in the response of host immune cells against *H. pylori*, which effectiveness depends on the polymorphism of those receptors[183]. Meta-analysis of *TLR2* -196 to -174 deletion and risk of gastric cancer conducted on 1364 gastric cancer patients and 2487 controls showed that there is an association between this polymorphism and risk of gastric cancer in the Japanese population. Polymorphism at this position decreases the induction of IL-8 secretion, thus impairing the response to *H. pylori*. Interestingly that correlation failed to be shown in the Chinese population, which may indicate an ethnic consideration in the incidence of stomach cancer[182].

CD14

CD14 molecule and TLR4 both participate in the recognition of LPS[189]. During *H. pylori* infection monocytes and macrophages have been shown to release IL-12 in response to CD14 - dependent activation. This was correlated with the infiltration of gastric mucosa with T helper 1 lymphocytes and the maintenance of chronic inflammatory response[190].

Two SNPs identified in the promoter region of the *CD14* gene: -260C/T (rs2569190 or *CD14* -159) and -561C/T (rs5744455), have been suggested to increase the susceptibility to gastric cancer[182,191]. The *CD14* -260 T allele had decreased affinity for the binding with DNA of transcription factors such as stimulatory proteins (SP) 1, SP2 and SP3 of which SP3 downregulates the activation of the cells by SP1 and SP2. Thus, the SP3 to SP1 and SP2 ratio might play an important role in the regulation of CD14 transcription[182,192,193]. Although an increased transcription activity of this allele has been demonstrated in monocytes with low levels of SP3 a direct correlation between

CD14 polymorphism and gastric cancer incidence still needs to be investigated[190].

NODs

The NOD-like receptors detect PAMPs localized intracellularly as well as cellular DAMPs released due to elevated stress conditions. These receptors are involved in the development of innate immunity, regulation of inflammatory response and programmed cell death. Among NODs the binding specificity of NOD1 and NOD2 is different. NOD1 binds γ-D-glutamyl-meso-diaminopimelic acid whereas NOD2 muramyl dipeptide[194,195].

During *H. pylori* infection NOD1 is engaged in the induction of NF-κB and activator protein 1 (AP-1), which are involved in cytokine synthesis and cell activation, thus triggering inflammatory response[190,196-198]. It has been shown that NOD1 regulates direct killing of *H. pylori* by antimicrobial peptides[199], enhances IFN-γ signaling in gastric epithelial cells during *H. pylori* infection, particularly with *cag*-PAI positive strains and exacerbates disease severity[198,200]. NOD2 induces pro-IL-1β and is necessary for the induction of NLRP containing protein 3 (scaffolidng proteins of inflammasomes) in *H. pylori*-infected dendritic cells[201].

Polymorphism among NOD receptors also has an impact on the rate of stomach cancer incidence. Wang *et al*[202], carried out a test on a group of 296 patients with gastric cancer and 160 healthy subjects in the Chinese population, which showed that the *NOD1* rs2907749 TT polymorphism reduced the likelihood of cancer of the stomach but *NOD1* rs7789045 TT increased the incidence of stomach cancer (especially in the case of the *NOD2* genotype rs7205423). An enhanced NOD1 expression was detected in *H. pylori* infected gastric mucosa. This might suggest that signaling *via* NOD1 determines gastric inflammation[202]. In general there is no association between *NOD1*/*NOD2* mutations and gastritis as well as gastric ulcer. However, association between the R702W mutation in the *NPD 2/CARD15* gene and gastric lymphoma has been found. The risk of gastric lymphoma is higher in those who carry allele T as compared to control individuals[200]. Companioni *et al*[203] have found a significant association between SNPs in *CD14*, *NOD2* and *TLR4*. This study revealed that genetic variation in NOD2 associates with nocardia gastric cancer while variation in CD14 is associated with cardia gastric cancer.

INFLAMMATION DRIVEN MALIGNANCY RISK

Cytokines

During *H. pylori* infection the immune and gastric epithelial cells respond by the secretion of cytokines (pro- and anti-inflammatory). The level of cytokines might depend on polymorphisms of the genes encoding specific cytokines including tumor necrosis factor (TNF)-α, IL-1, IL-8 and Il-10[204]. Genetic polymorphisms have been considered as factors increasing cytokine levels and susceptibility for cancer development due to hypochloridria[205].

IL-1

IL-1 (IL-1α and IL-1β), is a pro-inflammatory cytokine and IL-1 receptor antagonist (IL-1Ra) possess a natural anti-inflammatory activity. The initiation or the maintenance of inflammation depend on the balance between IL-1β and IL-1Ra[204]. IL-1β and IL-1RN gene polymorphisms increase risk of hypochloridria and gastric carcinoma. This is because the elevated levels of IL-1 initiate spontaneous inflammation, which then can be followed by dysplasia and gastric carcinoma through an activation of the IL-1/NF-κB pathway[206-208]. It has been shown that IL-1β significantly amplifies inflammatory response during *H. pylori* infections[204,205]. Ramis *et al*[204], investigated in the *IL-1B* gene three SNPs (C-T transition at -31 position; C-T transitions at -511 and +3954 positions), associated with an enhanced secretion of IL-1β. In *H. pylori* infected patients there was a correlation between IL-1β level and the T/T genotype (-511 position) as well as the C/C genotype (-31 position). In such patients an increased risk of gastritis but not peptic ulcer and gastric carcinoma has been found. This research group also proved that patients with the T/T genotype of *IL-1B* (-511 position) were more frequently infected with *H. pylori cagA*(+) strains. There was no correlation between *IL-1B* gene polymorphisms at position +3954 and increased prevalence of *H. pylori* infection as well as *H. pylori*-derived diseases[204].

However, in the Costa Rican population two proinflammatory genotypes *IL-1β*+3954 T/C and *IL-1RN**2/L were foud related to gastric cancer cases[209]. Caleman Neto *et al*[210], have suggested that the *IL-1β* -31T/T polymorphism acts as a protective factor against *H. pylori* infection in the Brazilian population.

Contrary to previous studies, Al-Moundhri *et al*[211], has proven that the widely reported association between *IL-1β* -31/-511 polymorphism and gastric cancer was not established in the Omani Arab population, supporting the ethnic differences in the effect of *IL-1B* polymorphism on gastric cancer development.

IL-1RN

Il-1RN as an antagonist of the IL-1 receptor modulates its activity. The most intensively studied *IL-1RN* polymorphism connected to gastric cancer outcome is a 86-bp variable number of tandem repeats polymorphism in the *IL-1RN* second intron (*IL-1RN**2)[208]. The study carried out on the Brazilian Amazon population by Melo Barbosa *et al*[205], showed that among patients with gastric ulcer and adenocarcinoma there was a higher frequency of allele 2 carriers (*IL-1RN*2*).

The IL-1Ra protein (encoded by the *IL-1RN* gene) competes with the IL-1 receptor to inhibit the action induced by IL-1β. The presence of the *IL-1RN*2* variant is connected with the increased levels of IL-1β in the gastric mucosa and to hypochlorhidria in comparison to *IL-1RN1/1* variant[205]. Research performed on a group of 118 gastric cancer patients and 245 healthy controls also supported the correlation between the presence of the *IL-RN*2* allele and the increase in the gastric cancer ratio in the Arab population[211].

Tumor necrosis factor alpha

TNF-α is a cell signaling protein involved in systemic inflammation and acute phase reaction. This cytokine is produced by activated macrophages, CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. It takes part in the regulation of immune cell activity, fever induction, apoptotic cell death, cachexia, inflammation, inhibition of tumorigenesis and viral replication. It is also involved in the cytokine response during sepsis[212,213]. The elevated secretion of TNF-α is observed in the gastric mucosa of *H. pylori* infected patients where this cytokine induces cell apoptosis[214]. The activity of TNF-α is regulated by soluble TNF receptors (sTNF-Rs), which potentially protect gastric epithelial cells colonized by *H. pylori* from apoptosis[214].

TNF-α activity and concentration can be influenced by SNPs (G to A transitions at -308A and -238 positions) in the promoter region of *TNF-α* gene[215]. In the Korean population the transition at -308 position was related with a CagA(+) *H. pylori* infections and its severe consequences. The biallelic polymorphism at this position is associated with the development of gastric carcinoma in the Caucasian population[205].

The binding of AP-2 to -308 region was found by Yea *et al*[215] to be altered by the −308A allele. Due to this -308A polymorphism might lead to an increase in *TNF-α* gene expression[215].

The latest results of meta-analysis obtained by Sun *et al*[216] demonstrate that *TNF-α* -308G/A and -1031 T/C polymorphisms may be protective factors against *H. pylori* infection, whereas -863C/A substitution may be a risk factor, especially in Asian populations. The authors also showed that there was no significant association between -857C/T polymorphism and *H. pylori* infection while -863C/A significantly increased the risk of infection. Moreover, the -1031T/C polymorphism decreased this risk for the Asian subgroup and hospitalized patients[216].

IL-10

IL-10 is a pleiotropic cytokine, which has the ability to suppress or stimulate anti-cancer properties of immune cells. This cytokine downregulates the production of pro-inflammatory cytokines by inhibition of Th 1 lymphocytes and stimulation of B, as well as Th 2, lymphocytes and thus downregulates the inflammatory response[212,213]. Since 2003 researchers have consistently reported associations between *IL-10-592* A/C SNP and susceptibility to gastric cancer but with mixed or conflicting results[217]. A meta-analysis performed by Ni *et al*[218] indicated that in Asian populations the carriers of *IL-10* -1082 GG-plus-GA genotypes are more susceptible to all types of gastric cancer.

Kim *et al*[219] investigated three *IL-10* promoter polymorphisms: -1082A/G, -819T/C, and -592 A/C probably related to elevated levels of IL-10. These polymorphisms were associated with an increased risk of intestinal-type noncardiac gastric cancer but only in *H. pylori* infected smokers[219].

Con *et al*[209] showed that the *IL-10* -592 A/A or -592 C/A polymorphisms were associated with an increased risk of gastric cancer in the Costa Rican population. In the above study the *IL-1β* +3954 T/C, *IL-1RN**2/L and *IL-10*: -592 C/A polymorphisms, in the patients infected with *H. pylori vacA s1b/m1* strains have been found to predispose them to gastric cancer. It means that synergistic effect of bacterial and host genotypes may influence the course and the consequences of *H. pylori* infection[209].

IL-8

During early phase of *H. pylori* infection a chemotactic IL-8 induces infiltration of granulocytes to the site of infection and induction of phagocytosis once they have arrived[204]. Activation of phagocytes in the inflammatory milieu may result in gastric barrier damage due to releasing of proteolytic enzymes and reactive oxygen radicals[220].

As in the case of other cytokine polymorphisms, IL-8 differentiation is also the subject of research. Caleman Neto *et al*[210] suggested that in Eastern populations the elevated production of IL-8 and the intensity of the inflammatory response depends on the presence of the A allele in the promoter region of the *IL-8 gene* (-251 position).

Ohyauchi *et al*[220] investigated a correlation between *IL-8* polymorphism and gastroduodenal disease outcome during *H. pylori* infection in the Japanese population. Thy showed that in *H. pylori* infected patients the presence of *IL-8* -251A allele was linked with the gastric ulcer, gastric atrophy and then cancer. This study confirmed that, in comparison to the *IL-8* -251T variant, *IL-8* -251A transcription is activated in more active gastritis with strong neutrophil infiltration. These results have been confirmed by the study of Caleman Neto *et al*[210], performed with 60 patients, which showed that the *IL-8* -251TT genotype could protect whereas the *IL-8* -251TA genotype could promote the *H. pylori* infection.

Cyclooxygenase-2

Cyclooxygenase-2 (COX-2) catalyzes the conversion of arachidonic acid to prostaglandins and its

production increases in response to growth factors, cytokines and mitogens. COX-2 is often undetectable in normal tissues, whereas in tumor tissue specimens its expression is higher[221,222]. Specifically, increased COX-2 expression is linked to the progression of gastric cancer and precancerous tissues by activating angiogenesis, inhibiting apoptosis, and accelerating invasion and metastasis[221]. In addition to cytokine polymorphisms, genetic differentiation of cyclooxygenase also plays an important role in the development of *H. pylori*-associated gastric diseases[222]. Concerning the polymorphisms of promoter region of *COX-2* (1195G/A and -765G/C), Li *et al*[222] showed that the increased risk of gastric cancer appears in the carriers of the *COX-2*-1195AA but not of the *COX-2*-765G/C genotype.

Meta-analysis carried out by Zhao *et al*[221] showed that the -765G/C polymorphism (rs20417) in the promoter region of the *COX-2* gene could be a risk factor for gastric cancer in Asians and Indians. This SNP affects the transcription and functional activity of COX-2. The *COX-2*-765G/C polymorphism was significantly associated with an increased risk of gastric cancer, regardless of *H. pylori* infection.

Polymorphisms involved in deregulation of T cell response

Gastric MALT lymphoma depends on the activation of specific T lymphocytes, which undergo regulation through different mechanisms. It depends on cytotoxic T-lymphocyte antigen (CTLA) 4 as well as CD28 and inducible costimulator (ICOS) genes[223-225]. Genotyping of *CTLA 4* gene (49 A/G, -318 C/T, CT60 A/G), *CD28* gene (IVS3+ 17T/C), and *ICOS* gene (c.602 A/C and c.1624C/T) has been performed by Cheng *et al*[226] in the gastric MALT lymphoma patients with or without *H. pylori* infection and healthy individuals. The *CTLA 4* -318 C/T genotype was associated with a lower whereas the *CTLA 4* 49 G/G genotype with a higher risk of MALT lymphoma. In *H. pylori*-positive patients, the susceptibility to MALT lymphoma was four times higher in the case of the carriage of -318C -49G haplotype.

CONCLUSION

H. pylori has evolved during long cohabitation with humans. The colonization of the host stomach at a young age, persistence in this specific niche for its lifetime, subversion of the human immune system by hypoinflammatory LPS and molecular mimicry, and induction of gastritis and cancer development make *H. pylori* a complex pathogen. The clinical aspects of *H. pylori* depend on several conditions, such as the location of infection, the host susceptibility, the bacterial strain and environmental factors. The virulence strategies of bacterial CagA-positive strains, as well as low socioeconomic status of the patient, influence the outcome of the infection. The highest prevalence rates of infection are reported in Asia and Africa. For years *H. pylori* infection might remain asymptomatic in spite of the developing condition. Medication for chronic gastritis or peptic ulcers involves antibiotic therapy. Sequential therapy is the most efficient treatment to cure the infection. To prevent the occurrence of antibiotic resistance, only cases with clinical symptoms or asymptomatic patients in a risk group ought to be treated. Adequate results for *H. pylori* detection are provided by the non-invasive urea breath test and invasive nested PCR. Eradication of the infection typically leads to improved patient health, but it may allow the development of gastroesophageal disease and asthma. The intensity of the infection reflects the ability of *H. pylori* to induce extragastric diseases. Chronic atrophic gastritis is the precursor condition for ulceration and gastric malignancy. Classified as a group I carcinogen and causing nearly 670 thousand new cancer cases every year, *H. pylori* has become a threat to our lives. Specific biomarkers are crucial for early diagnosis of gastric cancer. Although *H. pylori* is one of the most studied pathogens of the upper gastrointestinal tract, many of its mechanisms of action are still not well understood.

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REVIEW

Pseudopolyps in inflammatory bowel diseases: Have we learned enough?

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Abstract

Pseudopolyps are a well described entity in the literature and even though the exact pathogenesis of their formation is not completely understood, they are considered non-neoplastic lesions originating from the mucosa after repeated periods of inflammation and ulceration associated with excessive healing processes. Their occurrence is less common in Crohn's disease than in ulcerative colitis, and their overall prevalence ranges from 4% to 74%; moreover, they are found more often in colon but have been detected in other parts of the gastrointestinal tract as well. When their size exceeds the arbitrary point of 1.5 cm, they are classified as giant pseudopolyps. Clinical evaluation should differentiate the pseudopolyps from other polypoid lesions, such as the dysplasia-associated mass or lesion, but this situation represents an ongoing clinical challenge. Pseudopolyps can provoke complications such as bleeding or obstruction, and their management includes medical therapy, endoscopy and surgery; however, no consensus exists about the optimal treatment approach. Patients with pseudopolyps are considered at intermediate risk for colorectal cancer and regular endoscopic monitoring is recommended. Through a review of the literature, we provide here a proposed classification of the characteristics of pseudopolyps.

Key words: Pseudopolyps; Inflammatory polyps; Post-inflammatory polyps; Giant pseudopolyps; Ulcerative colitis; Inflammatory bowel disease; Crohn's disease; Classification; Dysplasia-associated mass or lesion

Core tip: In inflammatory bowel disease patients, pseudopolyps are formed at the bowel wall during the

inflammatory process. Published reports have begun to elucidate the mechanism of pseudopolyp formation and prevalence; however, the clinical challenge in distinguishing these entities from other dysplastic lesions remains and there is scarce data about their complications and management. In this review, we aimed to condense the published reports about their prevalence and to present a classification of their distinct characteristics based on endoscoping and histologic criteria, in order to facilitate their recognition. Moreover, available methods for confronting their complications and long-term management are presented.

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INTRODUCTION

The word *pseudopolyp* (PP) derives from the compound pseudo, a prefix with Greek origin meaning "fake", and a second compound, polyp, which means "any projection into the intestinal lumen above the layer of mucosa"[1]. The precise pathogenesis of these "fake" polyps is not entirely understood, even though a respectable number of reports exist in the current medical literature. PPs have been described in association with ulcerative colitis (UC) as far back as 1926, although the modern identifier terminology was not used at that time[2]. Their name originated as an effort to separate them from the true neoplastic polyps, namely adenomas[3].

This review focuses on the description of the distinct characteristics of PPs, emphasizing their management and differentiation from dysplasia-associated lesion or mass (DALM) encountered in inflammatory bowel disease (IBD) patients.

LITERATURE RESEARCH

To conduct this review, a search of the medical literature of the PubMed database was carried out to identify articles published up to January of 2016. Topically relevant articles were identified using the terms "pseudopolyps", "inflammatory polyps", "inflammatory pseudopolyps", "giant pseudopolyps"," post inflammatory pseudopolyps", "inflammatory bowel disease", "ulcerative colitis", "Crohn's disease", and "colonic polyps". Bibliographies of the relevant articles were manually searched to identify potentially topical supplementary references, which were retrieved and reviewed. The images provided in this review represent cases managed in our clinical division.

DEFINITIONS AND MECHANISMS OF FORMATION

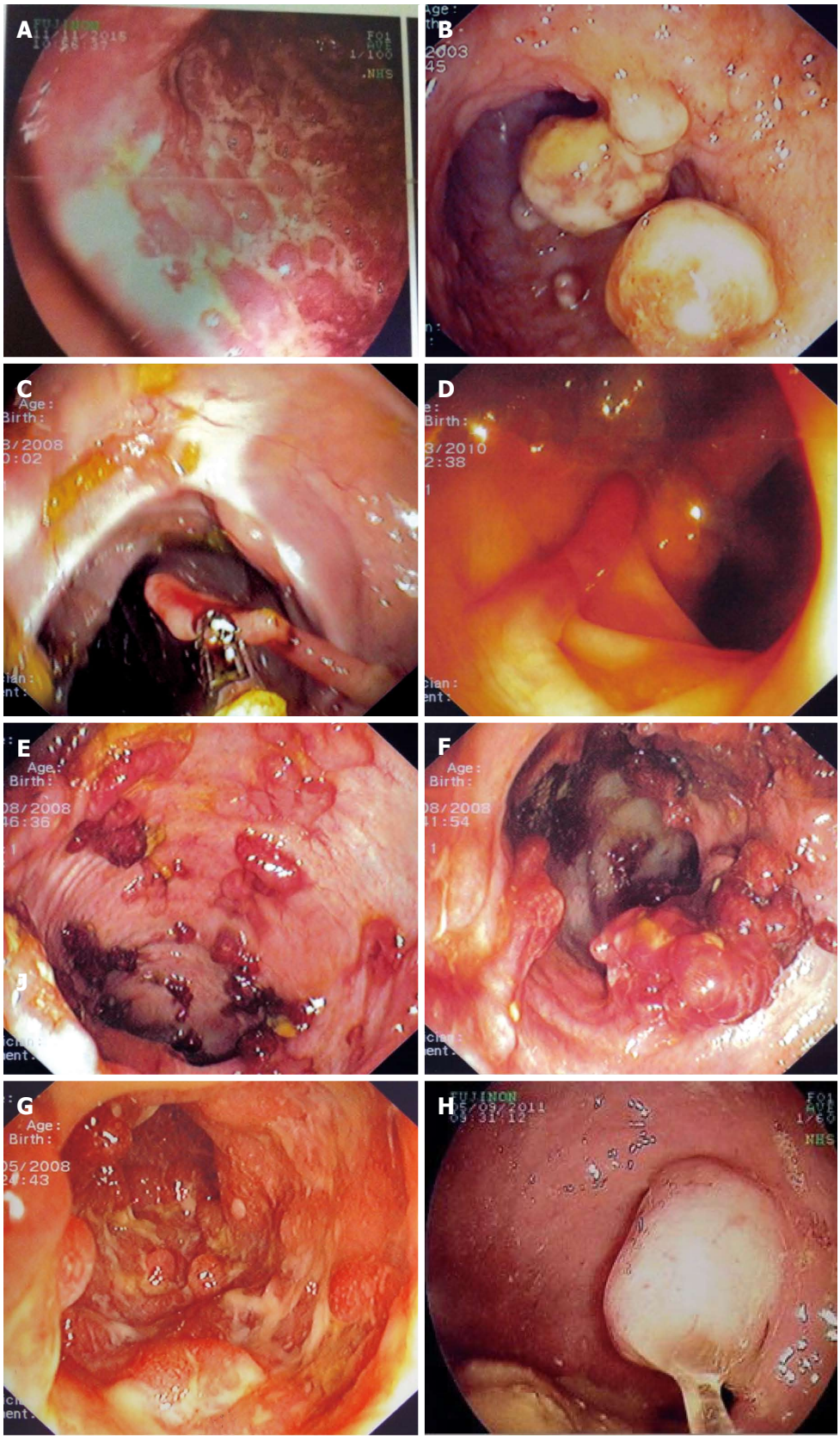
PPs are formed as a consequence of alternating cycles of inflammation and regeneration of the ulcerated epithelium[4]. The terms *pseudopolyps*[5], *inflammatory polyps*[6], *post-inflammatory polyps*[7] or *inflammatory pseudopolyps*[8] are often applied interchangeably in the literature, creating confusion. The term *pseudopolyps*, however, has been applied to the characterization of surviving islets of mucosa between ulcers during a severe attack, which create the impression of a polyp[9], and of loose mucosal tags, which are formed because of severe ulceration undermining the integrity of the muscularis mucosa. In conjunction with the inflammation process and cellular infiltration of the submucosa, granulation tissue is formed, which is more intense in some focal areas, thereby producing inflammatory polyps[10]. During the healing process, which features re-epithelization and excessive regeneration, post-inflammatory polyps are formed[11], taking their shape from the elongation of mucosal tags related to the bowel's peristaltic contractions and the stream of feces[12]. From this perspective, the post-inflammatory polyps can be separated into the following categories: (1) pseudopolyps; (2) inflammatory polyps; and (3) post-inflammatory polyps.

HISTOLOGY

Histology reveals the various aspects of inflammation-acute and chronic-that occur in bowel wall, often simultaneously and parallel in neighboring areas of the colon. The first type is composed only from mucosa, which can be relatively intact or edematous, representing mucosal remnants between zones of ulceration and which, for most authors, are considered the "true" PPs[10] (Figure 1A).

Inflammatory polyps consist of compact, non-epithelialized granulation tissue, representing a dense mixture of lymphocytes, plasma cells and mast cells predominantly but also includes neutrophils and eosinophils, all of which are detected as infiltrating the proper lamina of ulcerated epithelium. Post-inflammatory pseudopolyps are composed of a layer of normal or slightly-hyperplastic glandular epithelium, mucosa muscularis and a submucosa core of fibrovascular tissue. However, at the bowel wall, mixed forms of these types are frequently found; for example, remnant mucosa infiltrating granulation tissue or granulation tissue at the free ends of post-inflammatory polyps have been detected. The latter is due to secondary ulceration or inflammatory infiltration at the base of PPs[13].

Kelly *et al*[6] divided PP types into polypoid mucosal tags and mature inflammatory polyps, encompassing essentially all the previous forms, and proposed the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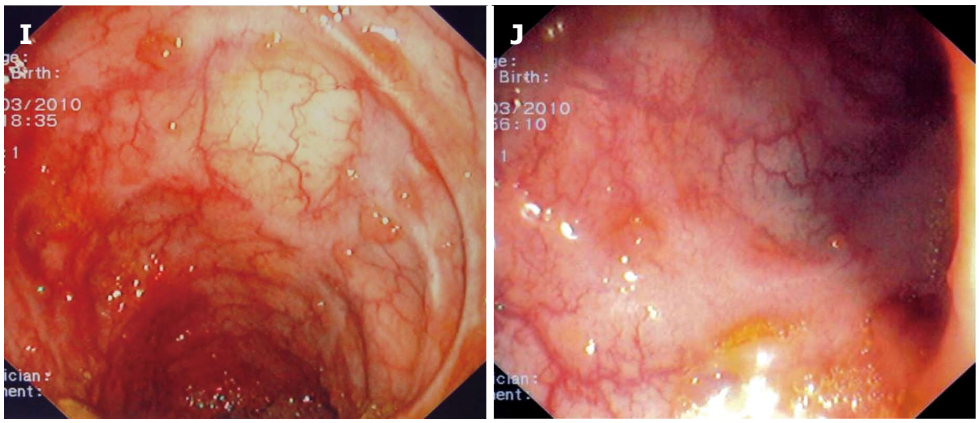

Figure 1 Examples of various types of pseudopolyps in different patients with inflammatory bowel disease. A: Endoscopic picture of deep ulcers and residual islets of surviving mucosa, the "true" pseudopolyps; B: Localized pseudopolyps of varying size up to 1.6 cm with discrete borders, pale surface, exudates on surface and varying forms. Biopsy of the polyps revealed inflammatory infiltration of lymphocytes, distortion and branching of the crypts compatible with post inflammatory pseudopolyps; C: Long filiform pseudopolyp located in the transverse colon captured with biopsy forceps; D: Localized filiform pseudopolyposis located in sigmoid colon; E: Post-inflammatory generalized pseudopolyposis; F: Cluster of pseudopolyps in sigmoid colon with 2.5 cm size, creating a giant localized pseudopolyp. Multiple biopsies of the polyp showed lined epithelium with a core of connective tissue and vessels with inflammatory infiltration which confirmed the diagnosis of pseudopolyp; G: Multiple pseudopolyps stubble the sigmoid colon. In this case, surveillance for dysplasia-associated lesion or mass can be challenging because of the intense inflammation and the multiple pseudopolyps; H: Pseudopolyp or adenoma-like mass? Solitary polyp with 1.5 cm size and broad-based with discrete borders but without exudates, amenable to endoscopic removal and having a pale surface. Histology after removal of the polyp with electrocautery showed villous adenoma with mild dysplasia, and without dysplasia in the surrounding mucosa or elsewhere in the colon, compatible with adenoma-like mass; I: Localized post-inflammatory pseudopolyps; J: Localized pseudopolyps of 0.3 cm maximum size, located in sigmoid colon with discrete borders and pale, glistering surface. Endoscopic characteristics were adequate for recognition of pseudopolyps without the need for biopsies or further intervention.

term *inflammatory polyp* as the most appropriate for general use. Histology of the giant pseudopolyp (GPP) type of PIP is composed of multiple bands of the same elements[5].

MORPHOLOGY AND DISTINCT FORMS OF PP

PPs exist in a variety of forms, including sessile, frond-like and pedunculated, and they can occur as solitary or multiple, or as diffuse or localized in distribution[9]. They also vary in size, but are usually short. When a PP exceeds 1.5 cm in size (Figure 1B), the term *giant pseudopolyp* has prevailed for their characterization[5], with this description first appearing in the literature in 1965[9].

A distinct form of the post-inflammatory polyps is the filiform polyps. These appear as slender, finger-like or worm-like projections of the mucosa and submucosa, and look like a polyp stalk without a head and often with branching[14] (Figure 1C and D). They often create a cluster, and as such are termed as *localized giant pseudopolyposis*[15] (Figure 1E and F). In the literature, the term *filiform polyposis* often accounts for GPPs[7]. Another distinct form of the post-inflammatory polyps is the bridged PP, representing a mucosal bridge that formed from a long filiform polyp connecting to the opposite end of the lumen[16].

LOCATION AND DISTRIBUTION OF PPS

PPs are more commonly encountered in large intestine, likely due to this tissue being affected in both UC and Crohn's disease (CD). The most common site is transverse colon and, thereafter, descending and sigmoid colon, with rectum being the least common site; moreover, PP in the rectum are usually found at the upper third region[17]. The GPPs show similar topographic occurrence[7]. However, as CD can involve the entire gastrointestinal tract, the PPs can be present throughout but have been detected less often in extra-colonic regions. There is an exception to this distribution pattern for UC patients with backwash ileitis, wherein PPs have also been found at the terminal ileum[18]. There are also reports of PPs located at the esophagus[19], stomach[20], and different parts of the small bowel[21], with ileum presentation predominating in the latter[22]. There is one case report of a CD patient with pansinusitis location of PP, which regressed with medical therapy[23], and another case report of a patient with refractory pouchitis who presented with a large PP located in an affected pouch[24].

PREVALENCE OF PP IN IBD

PPs are a common finding in IBD[13]. They are found more often in UC than in CD, and some authors have reported a double prevalence in UC as compared with colonic CD[25]. The reported prevalence rates vary from 4% to 74%[26,27], but most of the data supporting these findings was obtained from older studies that considered only UC. The most commonly reported incidence rates in UC fall within the range of 10%-20%[28]. This variation in reported prevalence can

Table 1 Prevalence of pseudopolyps in inflammatory bowel disease

Ref.	Year of publication	IBD diagnosis	Prevalence of pseudopolyps	Special characteristics
Bargen *et al*[29]	1929	UC (*n* = 693)	10.0%	
Baars *et al*[30]	2012	UC (*n* = 171)	30.0%	44% of UC patients and 30% of CD patients with unknown status for PP
		CD (*n* = 77)	38.0%	
Baars *et al*[31]	2012	UC, CD (*n* = 152)	20.0%	
Bacon *et al*[32]	1956	UC (*n* = 84)	57.1%	Colectomy specimens
Bockus *et al*[27]	1956	UC (*n* = 125)	74.0%	Hospitalized patients
Chang *et al*[21]	2007	CD (*n* = 23)	22.0%	Examined only small intestine
Chawla *et al*[26]	1990	UC (*n* = 50)	4.0%	
Chuttani *et al*[33]	1967	UC (*n* = 46)	15.0%	
De Dombal *et al*[17]	1966	UC (*n* = 465)	12.5%	
De Felice *et al*[19]	2015	CD (*n* = 24)	4.0%	Location esophagus
Dukes *et al*[11]	1954	UC (*n* = 120)	10.0%	Colectomy specimens
Edwards *et al*[34]	1964	UC (*n* = 624)	14.9%	
Geboes *et al*[39]	1975	CD (*n* = 43)	16.0%	
Jalan *et al*[10]	1969	UC (*n* = 399)	18.7%	
Kelly *et al*[6]	1987	UC, CD (*n* = 86)	UC: 36%	Colectomy specimens
			CD: 17%	
			GPP: 4.6%	
Lescut *et al*[35]	1993	CD (*n* = 20)	10.0%	Only small intestine examined as location
Luo *et al*[36]	2009	UC, CD (*n* = 34)	29.0%	Pediatric population
Maroo *et al*[37]	1974	UC (*n* = 122)	8.0%	
Modigliani *et al*[50]	1990	CD (*n* = 142)	41.0%	Active colonic or ileocolonic CD
Ray *et al*[38]	2011	UC (*n* = 40)	27.0%	
Rutter *et al*[44]	2004	UC (*n* = 136)	39.0%	Control population without CRC
				Population with CRC
Tandon *et al*[40]	1965	UC (*n* = 69)	17.6%	
Teague *et al*[41]	1975	UC (*n* = 150)	17.0%	
Teh *et al*[42]	1987	UC (*n* = 61)	21.3%	
Velayos *et al*[43]	2006	UC (*n* = 188)	42.0%	Control population without CRC
		UC (*n* = 188)	56.0%	Population with CRC
Wang *et al*[45]	2007	UC (*n* = 2726)	22.0%	Active UC
Watts *et al*[46]	1966	UC (*n* = 169)	47.0%	Surgical specimens
Waugh *et al*[47]	1964	UC (*n* = 205)	5.9%	Surgical specimens
Wright *et al*[48]	1965	UC (*n* = 269)	10.0%	
Zheng *et al*[49]	2007	CD (*n* = 27)	48.0%	

CD: Crohn's disease; CRC: Colorectal cancer; IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

be ascribed to miscellaneous diagnostic criteria and different populations studied[6,9-11,17,19,21,26,29-50] (Table 1). For the prevalence of GPP, in particular, a review of 53 colectomised patients with GPPs found that 66.6% had CD and 33.7% had UC[12]; however, a more recent review of 78 patients with IBDs and GPPs found a prevalence of 53.8% in UC patients, which was slightly higher than that found in CD patients (46.2%)[7].

There is similar prevalence of PPs in both sexes, and the peak overall incidence is at the ages between 20-40 years. There is no trend in increasing prevalence with extended period of history of the IBD. Specifically, Jalan *et al*[10] reported that 33% of patients with PP had a < 5-mo history of UC and De Dombal *et al*[17] reported that among 204 patients with UC, 8.8% had PP on the first flare. For cases of GPPs, Ooi *et al*[51] reported appearance with a median disease history of 5 years after diagnosis for UC and 6 years after diagnosis for CD; however, there was a broad variation in the times of appearance, ranging from 1 mo to 20 years for UC and from 3 mo to 37 years for CD.

CLINICAL SIGNIFICANCE

The presence of PPs in a patient with IBD can be an indirect marker of previous episodes of severe inflammation, and their incidence rises with more extensive colitis. Although there are not any clear prognostic criteria predicting their formation, it is a common belief that intense flares and hyperplastic healing predispose to PP formation. A cornerstone study by De Dombal *et al*[17], involving 465 patients with UC, has shown that 19.5% of patients with total colitis had PPs and 38% of the patients with PPs had suffered at least one episode of severe flare; in addition, 57.1% of the patients who underwent colectomy to address fulminant UC in 1956 had PP. This high prevalence can be attributable to severe active disease[32]. Teague *et al*[41] expressed a similar opinion, citing a PP prevalence of 41% in 48 patients with total colitis, and Jalan *et al*[10] reported that 31% of patients with severe UC had PP.

In regards to predicting PP formation, Babic *et al*[52] proposed that elevation in two of the three following

Table 2 Pseudopolyps and increased incidence of colorectal cancer

Ref.	Year of publication	IBD diagnosis	Format of study	Cancer risk
Rutter *et al*[44]	2004	UC with CRC (n = 68)	Case-control study 1:2, documentation of PP	OR = 2.29; 95%CI: 1.28-4.11
Velayos *et al*[43]	2006	UC with CRC (n = 188)	Case-control study 1:1, history of PP	OR = 2.5; 95%CI: 1.4-4.6
Baars *et al*[57]	2011	UC (n = 113) CD (n = 58) IC (n = 2)	Case-control study 1:2	RR = 1.92; 95%CI: 1.28 -2.88

CD: Crohn's disease; CRC: Colorectal cancer; IBD: Inflammatory bowel disease; IC: Intermediate colitis; OR: Odds ratio; PP: Pseudopolyp; RR: Relative risk; UC: Ulcerative colitis.

Table 3 Characteristics for differential diagnosis between pseudopolyps, adenoma-like DALM and non-adenoma-like DALM

	Pseudopolyps	Adenoma-like DALM	Non-adenoma-like DALM
Number	Often multiple	Can be multiple, usually solitary	Usually solitary
Location	Located in area inside colitis	Located in area inside and outside colitis	Located in area inside colitis
Endoscopic appearance	Smooth surface, can have exudate, definite borders, pale surface	Well circumscribed, definite borders, smooth surface sessile or pedunculated	Not amenable to endoscopic removal, irregular borders, often ulcerated or necrotic material
Management	No necessity for removal or biopsies except doubt	Endoscopic removal and endoscopic surveillance if dysplasia not recognized in adjacent mucosa or in other area of colitis	Proctocolectomy when HDG in lesion or multifocal LGD in area of colitis

DALM: Dysplasia-associated lesion or mass; HGD: High-grade dysplasia; LGD: Low-grade dysplasia.

parameters-C-reactive protein, C4 and procollagen Ⅲ peptide-accompany formation of PP in UC, calculating the positive predictive value and accuracy to be as high as 90% and 93%, respectively. The existence of PP has also been linked with the occurrence of extra-intestinal symptoms, specifically arthropathy[10]. Their presence in general, however, does not characterize any specific phase of IBD, as they can be found in both active and quiescent disease states, with the exception of the first form (*i.e.,* the mucosal remnants) which are only found in active IBD[53].

PP AND RISK FOR COLORECTAL CANCER

Patients with PP are considered to be at intermediate risk for colorectal cancer (CRC). United Kingdom guidelines suggest surveillance colonoscopy be performed at a 3-year interval[54], European Crohn's and Colitis Organization guidelines suggest colonoscopy at 2- or 3-year intervals[55] and the American Society for Gastrointestinal Endoscopy suggests between 1- and 3-year intervals[56]. Three studies, performed by Rutter *et al*[44], Velayos *et al*[43] and Baars *et al*[57], have shown a near 2-fold increased risk of CRC in patients with previous or present PP in endoscopy (Table 2). In much older reports, there was a debate about the possibility of PP malignant transformation, with advocates representing both sides. Among these, Goldgraber *et al*[9] reported a case series of several forms of PP with some showing premalignant changes, but later analysis proved these were benign lesions, regardless of size[10,34].

Nowadays, malignant transformation of PP is considered an extremely rare event, with only two reports of GPP harboring carcinoma or dysplasia features[11,58]. Another case report from Klarskov *et al*[59] presented a carcinoma in rectum stump that had arose from serrated adenoma with a filiform form. The authors speculated that the serrated adenoma had derived from transformation of preexisting PP. A possible mechanism has been implicated by Jawad *et al*[60], who reported that PP can be the source of premalignant mutations, following their analysis of DNA taken from 30 PP samples and which showed four identifiable mutations. However, more studies are needed to confirm the doubt in their benign nature.

A possible explanation about the relationship between PP and increased risk of CRC lies in the facts that they are considered markers of episodes of previous severe inflammation and that their incidence of appearance rises with the increased extent of colitis[17], which is in turn linked to CRC. Another possible explanation is that their presence, especially if they are numerous, can obscure the capability of finding dysplastic lesions in endoscopic surveillance[43] (Figure 1G).

LONG-TERM MANAGEMENT

Questions remain about the optimal management or follow-up strategies for PP, especially for cases with multiple PP, because no large trials have been published regarding these issues. A great matter of concern involves distinguishing them from adenoma-like DALM and non-adenoma-like DALM (Figure 1H). The main characteristics and differences between these entities are summarized in Table 3, and include fea-

tures such as location and endoscopic appearance[61-64]. Even though some diagnostic endoscopic criteria may be used for recognizing PP, they are not completely reliable[65]. There can be good inter-observer agreement for identifying PP during endoscopy in general[66], but when it comes to distinguishing PP from other dysplastic lesions, the efficiency falls. Farraye *et al*[63] performed an internet-based study and found that gastroenterologists with non-IBD-specialized expertise had lower capability of distinguishing different forms of lesions in IBD patients.

There is a general acceptance that if PP are adequately recognized using endoscopic criteria and do not provoke any complications, no removal is considered obligatory[63] (Figure 1I and J). However, it is considered mandatory that the surface of any PP be surveyed adequately during endoscopy. In older reports, especially of cases with large PP, surgical intervention was frequently performed for the removal, due to confusion with CRC or villous adenoma and related to the more common use of radiological approaches, such as barium enema, for diagnosis and monitoring[67]. Nowadays, however, endoscopic surveillance is more effective than surgical intervention[51].

Chromo-endoscopy might aid in differential diagnosis, since PPs (as non-neoplastic polyps) show Kudo's pattern classification of type Ⅱ[68]. In another study by Koinuma *et al*[69], magnifying endoscopy was demonstrated as a useful tool for distinguishing neoplastic from non-neoplastic lesions, reducing the amount of biopsies needed; however, the efficacy of this technique for studying the underlying inflammatory process was shown to be limited by the presence of multiple PPs[65]. In another study, 165 patients with long-standing UC were divided and randomized for endoscopic surveillance by means of either conventional endoscopy (with biopsies every 10 cm) or chromo-endoscopy (with 0.1% methylene blue); there were two false-negative results that were not identified by the chromo-endoscopy procedure, for which non-targeted biopsies from colons with multiple PP proved to contain dysplasia[70].

Nevertheless, in cases where there is either doubt about the diagnosis of PP, suspicion of DALM or large-size PP, or presence of multiple PP wherein endoscopic surveillance is compromised, multiple biopsies should be obtained in repeated examinations[56,71] or proceeding the endoscopic or surgical removal, with surrounding tissue examination by biopsy as well[72].

In the same context, the discovery of PP in a patient with IBD, without evidence of suspicious lesions in endoscopy and in which the presence of PP does not obstruct adequate endoscopic surveillance of the mucosa, should not urge gastroenterologists towards more intense endoscopic follow-up. Neither should it discourage them from the use of chromo-endoscopy for surveillance in any manner other than those proposed in the various guidelines (with an approximate 3-year interval), and certainly not in a different way than would be performed in patients without PPs[54-56]. As mentioned before, CRC derived from PP is a rare event and occurrence of PP has not been linked with early CRC[30]. Therefore, screening for CRC in all patients with PP is not recommended before 8-10 years after onset of symptoms[54-56].

COMPLICATIONS

In rare instances, PP can provoke serious complications, and physicians should be aware of these. Many reports have appeared regarding this issue for cases of GPP. Maggs *et al*[7] reviewed 78 patients with GPP, among which 15% were complicated with obstruction and sub-obstruction and 3% with intussusception of mechanical etiology due to the large size. In patients with CD, obstruction can occur in the small intestine with PP. In addition, GPPs can produce symptoms similar to IBD, such as bloating, diarrhea and abdominal pain. In that same review, from among the total of 25 patients with inactive disease, 11 had symptoms that regressed after removal of the GPP. Yet, it is important to emphasize that, even in cases of PP, the onset or persistence of symptoms cannot always be attributed to flare or activity of IBD.

There are reports of patients with generalized PP suffering from protein-losing enteropathy and pulmonary embolism, with the possible mechanism being extreme gastrointestinal losses due to the extensive inflamed surface area[73]; other complications include bleeding[74], iron deficiency anemia[75] and dysphagia[76].

TREATMENT

Treatment can be categorized as medical, endoscopic and surgical. Most reports dealing with complications have presented the use of interventional methods, but the majority of these are case reports. Medical treatment has been used for PP and shown to induce regression. Choi *et al*[71] reported regression of GPP in patients with IBD upon administration of mesalazine and azathioprine. Infliximab has also been shown to induce regression of PP in CD[77]. Topical enema with budenoside use was also reported to induce remission and control of minor bleeding of PP in sigmoid colon[78].

Endoscopic procedures such as argon plasma coagulation[79], endoscopic loop polypectomy[80], and ablation with yttrium aluminium garnet (commonly referred to as YAG) laser have been reported for control of bleeding provoked by ulcerated PP[81]. Endoscopic resection with electrocautery is another effective means reported for removing either symptomatic PPs or PPs of which their benign nature was not able to be established only with endoscopic criteria and which need further histological evaluation[82].

Surgical methods are used when endoscopic therapy fails to manage complicated PP, for example

Table 4 Summary of characteristics of pseudopolyps and other polypoid lesions in inflammatory bowel disease

Pseudopolyps and polypoid manifestation	Characterization
Location	Upper gastrointestinal tract
	Small bowel
	Large bowel
	Both small and large intestine
	Special location (pouch)
Size	< 1.5 cm
	> 1.5 (giant)
Number	< 10
	> 10 multiple
Pattern of distribution	Congested
	Scarce
Years since disease onset	< 1 yr
	1-5 yr
	> 5 yr
Bowel background mucosa	Relapsed
	Remission
Endoscopic appearance	Obstructing
	Bridging (mural bridging lesions)
	Penduculated
	Filiform (digitiform or fingerlike)
	Flat
	Mixed type (> 2 types of previous categories)
	Long, glistering, with or without exudate
	Resectable or not
	Definite borders, not stricturing
Histology	Inflammatory
	Adenomatous
	Dysplastic low-grade (DALM)
	Dysplastic high-grade (DALM)
	Serrated
IBD type	Ulcerative colitis
	Crohn's disease
	Indeterminate colitis
Follow-up	Reduction in number
	Reduction in size
	Increase in number
	Increase in size

DALM: Dysplasia-associated lesion or mass; IBD: Inflammatory bowel disease.

in lower gastrointestinal bleeding or when obstructing phenomena, such as luminal obliteration or intussusception, occur[67]. The various surgical procedures range from segmental dissection to hemicolectomy[83], depending on the cause. However, with the recent advances in endoscopic treatment, the need for a surgical approach has lessened over time.

CONCLUSION

We have reviewed the main aspects regarding PPs and their pathogenesis, management and differentiation from DALM in IBD. Further research can focus on prognostic factors related to their formation. Another interesting subject for clarification is the relationship and comparison between different medical treatments and the possibility of reducing PP prevalence with the additional aim of changing the natural history of IBD.

A key question that remains is: Is the presence of PP a marker of more aggressive IBD with more flares? Theoretically, the answer is positive, accepting the fact that PPs are a result of severe attack. However, that answer leaves open the next question as to whether these patients are indeed suffering from more flares. In addition, it remains unknown whether the newer biological agents and intensified medical therapy, which potentially reduce PP formation, correspond to a decline in CRC risk. We believe that in order to facilitate the management of patients with PP and promote future research on this clinical topic, better documentation of characteristics of pseudopolyps in patients with PP is needed. To this end, Table 4 summarizes the information on descriptions of the characteristics of PPs, which we recommend should be documented when a patient with PPs is encountered.

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REVIEW

Current knowledge on the laboratory diagnosis of *Clostridium difficile* infection

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Abstract

Clostridium difficile (*C. difficile*) is a spore-forming, toxin-producing, gram-positive anaerobic bacterium that is the principal etiologic agent of antibiotic-associated diarrhea. Infection with *C. difficile* (CDI) is characterized by diarrhea in clinical syndromes that vary from self-limited to mild or severe. Since its initial recognition as the causative agent of pseudomembranous colitis, *C. difficile* has spread around the world. CDI is one of the most common healthcare-associated infections and a significant cause of morbidity and mortality among older adult hospitalized patients. Due to extensive antibiotic usage, the number of CDIs has increased. Diagnosis of CDI is often difficult and has a substantial impact on the management of patients with the disease, mainly with regards to antibiotic management. The diagnosis of CDI is primarily based on the clinical signs and symptoms and is only confirmed by laboratory testing. Despite the high burden of CDI and the increasing interest in the disease, episodes of CDI are often misdiagnosed. The reasons for misdiagnosis are the lack of clinical suspicion or the use of inappropriate tests. The proper diagnosis of CDI reduces transmission, prevents inadequate or unnecessary treatments, and assures best antibiotic treatment. We review the options for the

laboratory diagnosis of CDI within the settings of the most accepted guidelines for CDI diagnosis, treatment, and prevention of CDI.

Key words: *Clostridium difficile*; Toxigenic culture; Nucleic acid amplification tests; Enzyme immunoassay; Diagnosis; Glutamate dehydrogenase

Core tip: This work is a review of the strategies that may be used for laboratory diagnosis of infection with *Clostridium difficile*. First, we provide general recommendations for testing of samples taking in account the guidelines of the Society for Healthcare Epidemiology of America/Infectious Diseases Society of America and the American College of Gastroenterology. We reviewed diverse methods of diagnosis including, culture, toxigenic culture, cell cytotoxic neutralization assay and the use of enzyme immuno assays. Finally, we present an overview of singleplex and multiplex nucleic acid amplification tests.

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INTRODUCTION

Clostridium difficile (*C. difficile*) is a Gram-positive and strictly anaerobic bacterium that may exist in a vegetative form that is very sensitive to oxygen. During stress, *C. difficile* produces spores that enable the microbe to survive harsh conditions for prolonged periods of time and facilitate its dissemination in the environment[1]. Upon ingestion, the spores resist the low pH of the stomach and reach the anaerobic environment of the gut. When the intestinal microbiote is altered because of antibiotic treatment, especially broad-spectrum antibiotics, the spores germinate. Next, *C. difficile* develops into its vegetative form, proliferates, and colonizes the gut[2]. *C. difficile* infection (CDI) is the principal cause of antibiotic-associated diarrhea. Diarrhea because of CDI may be self-limited, mild, or severe, and is one of the symptoms of a variety of clinical syndromes due to CDI. Complications of CDI are pseudomembranous colitis, fulminant colitis, and toxic megacolon[3].

CDI is an intestinal disease mediated by potent cytotoxic enzymes that damage the intestinal mucosa[4,5]. These cytotoxic enzymes, toxin A (TcdA) and toxin B (TcdB)[6,7], alter cytoskeletal actin, which leads to diminished transepithelial resistance, fluid accumulation, and destruction of the intestinal epithelium[5,8]. *C. difficile* toxins also induce the release of pro-inflammatory cytokines from enterocytes, mast cells, and macrophages[9]. The genome of toxigenic strains of *C. difficile* has a pathogenicity locus (PaLoc) of 19.6 kb that contains the *tcdA* and *tcdB* genes. Other PaLoc genes are *tcdR* and *tcdC*; the former encodes a positive regulator and the latter a negative regulator of the expression of the A and B toxins. Yet another PaLoc gene is *tcdE*, which encodes a holin-like protein that may be involved in the secretion of toxins[10]. Besides, some strains produce the *C. difficile* binary toxin (CDT), which is composed of an enzymatic component, CdtA, and a binding component, CdtB. These components are codified by *cdtA* and *cdtB* genes, which are located in the CDT locus (CdtLoc). CDT may potentiate the toxicity of TcdA and TcdB and lead to a more serious illness and could, therefore, be considered another virulence factor[11,12].

The vast majority of diarrhea cases are not related to a particular pathogen. From all the stool samples submitted to the laboratory for testing of *C. difficile* toxins, only 10% to 25% are positive[13]; most commonly in cases of antibiotic-associated diarrhea. Other pathogens that may cause antibiotic-associated diarrhea are: *Staphylococcus aureus*, *Clostridium perfringens*, *Salmonella* species, and *Klebsiella oxytoca*[14]. The clinical suspicion of CDI is the presentation of diarrhea after administration of antibiotics shortly after the beginning of treatment and up to 8 wk after treatment initiation[13]. In mild to moderate disease, diarrhea is the main symptom and passing of watery stools with foul odor is characteristic. The presence of hematochezia is rare. Moderate to severe disease is usually accompanied by systemic symptoms such as abdominal cramps, fever (up to 40 ℃), leukocytosis (up to 50000 cells/mm^3), and hypoalbuminemia (< 2.5 mg/dL)[13,15]. Colitis is characterized by fever, cramps, leukocytosis, and the presence of leukocytes in feces. Furthermore, a thickened colon wall is observed with computed tomography and in half of the cases pseudomembranes can be seen with endoscopy[13]. When pseudomembranes are found, a diagnostic of CDI can be made, as antibiotic-associated diarrhea due to other pathogens tends to have normal endoscopy findings[16]. In severe cases, CDI may progress to toxic megacolon with the risk of colon wall perforation[15]. The diagnosis of toxic megacolon is accomplished through radiological evidence of colonic dilatation, commonly involving the ascending or transverse colon[17]. According to the most commonly used criteria for the diagnosis of toxic megacolon[18], three of the following four criteria should be present: fever, tachycardia, leukocytosis, and anemia. Additionally, dehydration, electrolyte disturbance, and hypotension or changes in mental status (any of the criteria must be present)[17-19].

Ever since *C. difficile* was recognized as the caus-

ative agent of pseudomembranous colitis, the number of CDI cases has increased worldwide. Recently, Lessa *et al*[20] estimated that the number of CDI in the United States was 453000, from which 29000 died within 30 d after diagnosis. The healthcare costs related to CDI were estimated to be $ 4.8 billion for acute care facilities alone[20]. Since the year 2000, both the number and severity of CDI have increased due to the emergence of a more virulent strain with a higher antimicrobial resistance[21]. After strain typing by pulsed-field gel electrophoresis (PFGE), restriction endonuclease analysis, and ribotyping, this strain was denominated BI/North American PFGE type 1 (NAP1)/027[21]. So far, all BI/NAP1/027 isolates are positive for binary toxin CDT and have an 18-base pair deletion in *tcdC* that is associated with an increased production of toxins A and B; a single base pair deletion at position 117 of *tcdC* has also been related to higher toxin expression[3]. Concerning antimicrobial resistance, BI/NAP1/027 are resistant to fluoroquinolones, which provides a selective advantage to this strain[21]. This strain is disseminated worldwide; the above is evidenced by reports from America, Europe, Asia, and Oceania[22].

One of the key points when treating CDI is to discontinue unnecessary therapy with antibiotics; thus allowing the gut microbiota to recover. The Clinical Practice Guidelines for *C. difficile* Infection in Adults of the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA), the Guidelines for Diagnosis, Treatment, and Prevention of *C. difficile* Infections of the American College of Gastroenterology (ACG) and the Guidance Document for *Clostridium difficile* of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) recommend the use of metronidazole when treating an initial episode of mild to moderate CDI. The dosage is 500 mg orally, 3 times per day for 10 to 14 d. In case of severe initial CDI, vancomycin (125 mg orally, 4 times per day for 10 to 14 d) should be administered[23-25]. A combination of oral vancomycin (500 mg 4 times per day) and intravenous metronidazole (500 mg every 8 h) are indicated for the treatment of severe, complicated CDI according to the SHEA/IDSA and ESCMID guidelines[23,25]. On the other hand, the ACG recommends a dosage of 125 mg of vancomycin. When treating a first episode of recurrence, the regimen should be the same as an initial case, according to the severity of the infection. If there is a second episode of recurrence, a pulsed regimen of vancomycin is recommended. Fidaxomicin is narrow spectrum macrocyclic antibiotic, approved by the American Food and Drug Administration (FDA), that selectively eradicates *C. difficile* with a minimum effect on the intestinal microbiota. The relapse rate of fidaxomicin is lower than the one of vancomycin[26-28].

Despite the high burden of CDI and the increased interest in the disease, episodes of CDI are often misdiagnosed. A study from Spain revealed that two out of three CDI cases were either undiagnosed or misdiagnosed[29]. The main explanation may be the lack of clinical suspicion, particularly in community cases with patients who do not meet the risk criteria (age > 65 years or previous hospitalization). Besides, an inadequate test may yield false-negative results[30]. Furthermore, the interpretation of laboratory data is complicated, as the presence of *C. difficile* in stool does not mean CDI; the other way round, the absence of *C. difficile* toxins does not rule out the possibility of CDI. To interpret laboratory results, the techniques that were applied must be considered. A correct diagnosis of CDI is important because it has a substantial impact on case managment, mainly with regards to antibiotic regimens. The diagnosis of CDI is primarily based on the clinical signs and symptoms and is only confirmed by laboratory testing[31]. Misdiagnosis has two main consequences: first, patients may be undertreated or overtreated; second, the delay of proper infection control allows for further dissemination[32].

The following is a review of the strategies that may be used for laboratory diagnosis of CDI. To date, the Clinical Practice Guidelines of the SHEA/IDSA provide one of the most widely acceptable guidelines for the diagnosis and clinical management of CDI cases[23]. Also, the Guidelines of the ACG focus on the recommendations for the diagnosis and management of patients with CDI as well as for the prevention and control of outbreaks[24]. The above documents provide useful recommendations on which this review is based.

GENERAL RECOMMENDATIONS

Recommended samples

Both the Clinical Practice Guidelines of the SHEA/IDSA and the Guidelines of the ACG state that *C. difficile* testing is recommended only for stool samples from patients with diarrhea (Table 1), which is defined as the evacuation of loose stools, three or more times in 24 h or less[23,24]. The ESCMID recommends testing only stools of Bristol score 5 to 7[25]. The Bristol scale is a graded visual scale, composed of seven grades which range from stools with a form of separate hard lumps (score 1) to watery stools (score 7)[33]. To correlate the Bristol scale with *C. difficile* detection, stool samples with Bristol scale $\geqslant$ 5 were tested for *C. difficile* with an enzyme immunoassay (EIA) for glutamate dehydrogenase (GDH) and toxins A/B followed by a molecular assay for indeterminate results[34]. Detection of *C. difficile* was more frequent in semiformed stools (Bristol 5 or 6) than in watery stools (Bristol 7). Bristol 5 stool specimens accounted for the highest rate of positive testing. Therefore, Bristol 5 stool specimens should not be discarded. Further study is required to verify whether specimen with lower Bristol scores should be tested. There was no association between the Bristol score and the rates of hospital-onset CDI, severe CDI, and complications of CDI[34].

Commonly, perirectal swabs are not accepted for

Table 1 General recommendations for *Clostridium difficile* testing

Recommendation	Ref.
C. difficile testing is recommended only for stool samples from patients with diarrhea	[23,24]
The testing of asymptomatic patients is not recommended	[23]
Perirectal swabs are not accepted for *C. difficile* testing unless the patient has developed ileus	[23,24]
Repeated testing of *C. difficile* does not improve detection and does not change the result	[23,24]
Retesting, as a proof of cure, remains controversial	[23,24]

C. difficile: *Clostridium difficile*.

C. difficile testing, except for selected cases, such as patients with ileus[23,24]. Ileus is characterized by a lack of bowel movements that causes a blockage of the intestines. For patients with ileus, an accurate sampling technique is the perirectal swab[35]. Some patients with CDI develop fever and abdominal pain but not diarrhea. These patients may develop severe complications, like fulminant colitis and intestinal perforations[36].

Patients to evaluate

The Clinical Practice Guidelines of the SHEA/IDSA recommend no testing of asymptomatic patients[23] (Table 1) because asymptomatic colonization with a toxigenic strain of *C. difficile* is common. But even samples from patients with persistent diarrhea may yield false-positive CDI results; for example, alternate etiology was reported to be the cause of the symptoms in 25% of a cohort of 117 cases that had been diagnosed with recurrent CDI[37]. Particularly, highly sensitive molecular assays may yield false-positive results. Positive CDI tests for asymptomatic patients are common. The carriage rates of toxigenic *C. difficile* strains are similar between open populations (6.6%)[38] and hospitalized ones (8%)[39]. Hospitalized populations tend to be at an increased risk of developing CDI due to antibiotic treatment and prolonged exposure because of long stays at healthcare facilities[40]. The environment and skin of asymptomatic carriers have higher percentages of spores and represent a permanent source of contamination and spreading of spores to other patients and setting surfaces. The continual washing of hands of both personnel and patients is a universal preventing measure[41].

The carriage rate of *C. difficile* is high among infants (0 to 3 years of age). Among 85 healthy infants at day nurseries, the carriage of *C. difficile* was 45%, and the frequency of toxigenic strains was 13%[42]. One-year follow-up studies among newborns revealed that 74% to 100% had CDI-positive stool, often in the neonatal period[42] In a study that followed 10 infants, 81/111 samples (73%) were positive for *C. difficile* and 21 (26%) had toxigenic strains[42]. Another study that followed 42 infants found that 106/288 stool samples (37%) were CDI positive[43]. Most strains (71%) were toxin producers. Interestingly, six infants evacuated loose stools during the study, but only three of them could be linked to *C. difficile*. Furthermore, carriage of *C. difficile* was similar in infants suffering from loose stools than children with normal stools.

A point to consider when testing specimens from infants is the difficulty to differentiate a diarrheal stool from a normal stool, since infant stool may not be fully formed. When comparing infant cases (age $\leqslant$ 12 mo) with CDI-positive diarrheal stool with cases with CDI-negative diarrheal stool no differences in clinical symptoms were found. However, in both groups, alternative causes of diarrhea were found[44]. Rather than looking for *C. difficile*, the authors suggested looking for other causes, especially viral ones, of diarrhea in infants.

Retesting of samples

Neither the Clinical Practice Guidelines of the SHEA/IDSA nor the Guidelines of the ACG recommend retesting (Table 1). This recommendation is especially valid for molecular methods. Several studies have demonstrated that repeated testing for *C. difficile* does not improve detection nor change the result. Repeated testing, particularly with molecular tests, only increases healthcare costs and the probability of false-positive results. After having implemented a new policy that alerted physicians about the consequences and disadvantages of a *C. difficile* PCR test within 7 d of an initial test, a healthcare institute saw the requests for CDI retesting reduced by 91%[45]. Among the 135 retests that were performed, 122 were repeaters after a negative initial test, and only 4 of them turned positive upon repeating the test. Even lower positive conversion rates (0.05%-1%) have been reported by others after repeating PCR assays on 4213 samples with negative results in a previous test[46]. Thus, an initial test with a negative test result does not justify retesting unless there is a founded suspicion of a false-negative result.

Retesting to monitor response to treatment also remains controversial. Although a negative conversion rate of 67.6% 14 d after a positive Cepheid Xpert *C. difficile* test has been reported[47], cases that were clinically cured remained positive when testing for toxins[48].

DIAGNOSTIC TESTS

Culture

A stool culture is essential to prepare isolates for molecular typing, which is required for epidemiologic studies. The SHEA/IDSA guidelines recommend toxigenic culture (TC) as the standard to which other methods should be compared. The first step is to recover *C. difficile* spores. Stool samples are either heated to 80 ℃ or mixed with an equal volume of

absolute ethanol and incubated at room temperature. This way vegetative cells and contaminating microbes are eliminated while spores are recovered[49]. Next, the sample is inoculated into a differential and selective medium that allows the spores to germinate. A well-known medium to recover *C. difficile* from stool specimens is cycloserine-cefoxitin-fructose-agar (CCFA)[50]. Cycloserine and cefoxitin are present at concentrations that inhibit the growth of most Gram-negative and Gram-positive bacteria, without affecting the growth of *C. difficile*. Fructose is an important nutrient and neutral red is added as a pH indicator. A 48-h solid culture of *C. difficile* in CCFA presents flat, grayish, and shiny colonies with spreading edges and a typical horse manure smell. Under ultraviolet light, the colonies appear yellow-green fluorescent. Under the microscope, cells of *C. difficile* are Gram-positive and possess subterminal to terminal spores. Identification of *C. difficile* colonies may be based on colony morphology, Gram staining, and odor; confirmation of species may be assessed trough biochemical systems for the identification of anaerobes.

To improve both the recovery and identification of *C. difficile* cultures, the original CCFA formulation has been modified. The addition of biliary salts, particularly sodium taurocholate, promotes germination[51] and the inclusion of egg yolk allows to verify lecithinase and lipase activity of isolates[50,52]. Furthermore, enrichment broths have been formulated to recover small amounts of spores and allow them to germinate. When comparing two broths, a cycloserine-cefoxitin fructose broth proved to be more sensitive than a cycloserine-cefoxitin mannitol broth that had been supplemented with taurocholate and lysozyme, (CCMB-TAL), but CCMB-TAL yielded better recovery rates when cultures were semi-quantified[49].

Furthermore, chromogenic media have been developed. For example, *C. difficile* grown on ChromID *C. difficile* agar (bioMérieux, France) yields black colonies that often can be observed after 24 h of incubation[53]. However, lengthening the incubation from 24 to 48 h increased the sensitivity significantly from 53% to 100% ($P < 0.001$)[54]. ChromID *C. difficile* agar yields a higher 24-h recovery (sensitivity, 92%) than CCFA (sensitivity, 22%)[55]. Likewise, ChromID *C. difficile* agar, which had a sensitivity of 100% and a recovery of 94%, outperformed CCFA supplemented with sodium taurocholate, which had a sensitivity of 87% and a recovery of 82%[56]. Yet another study confirmed that ChromID *C. difficile* agar performs best when compared to CCFA, cycloserine-cefoxitin-egg-yolk agar and tryptone soy agar with sheep blood[49].

Toxigenic culture

TC is a two-step reference method for the diagnosis of CDI. In step one, *C. difficile* strains are isolated and grown on a selective medium, and in step two, colonies are tested for toxin production on a variety of cell lines. The grown isolates are re-cultured in broth, and the supernatant is filtered and added to a cell line culture. The cytopathic effect (CPE) is evaluated and neutralized by antitoxin. This procedure may take a few days to accomplish, which makes it an impractical option for routine diagnosis[57]. Alternatively, testing of toxin production may be performed using an EIA[58,59].

The main concern about TC is the possibility of recovering non-toxigenic strains (strains that are not capable of produce toxins A and/or B). Another possibility is, though recovering a toxigenic strain, it may not be producing toxins, thus not causing clinical symptoms. When evaluating the clinical significance of TC and the citotoxicity assay on 169 samples that met CDI criteria, it was found that cases positive for both assays were more severe than cases that were positive for TC only. On the other hand, if only the cytotoxicity assay had been performed, one-third of the cases would have been missed[60]. The latter is an argument in favor of TC for CDI diagnosis.

Cell Cytotoxic Neutralization Assay

The Cell Cytotoxic Neutralization Assay (CCNA) has been considered the gold standard for the diagnosis of CDI. In this assay, the filtrate of a recently obtained stool sample is inoculated onto various sensitive cell lines to evaluate the CPE of *C. difficile* toxins, particularly TcdB[57]. CPE is observed as cell rounding; some strains may induce protrusions in the cell lines, a phenomenon known as "sordellii-like" CPE[61,62]. If the CPE can be reversed by an antitoxin, the test is positive for the *C. difficile* toxin[57]. The assay must be performed in fresh stools, since sample freezing or a delay in its processing may result in loss of activity of toxins and false negative test results[63].

Diverse cell lines have been used for the detection of toxins: African green monkey kidney, McCoy, MRC-5, primary rhesus monkey kidney, and Vero cells. Among these cell lines, Vero cells and McCoy cells were a 100% concordant with respect to the detection of toxins[64]. When maintaining a cell line is impossible or non-desirable, there are cost-effective commercial assays available. A CCNA executed with Hs27 HFF ReadyCells is not only easy-to-use but also outperforms the EIA and TC in both sensitivity and specificity; 90.8% *vs* 78.6% in sensitivity and 98.3% *vs* 97.8% in specificity, respectively[65].

Glutamate dehydrogenase assay

C. difficile produces and secretes GDH; this enzyme allows the bacterium to manage oxidative stress derived from the immune response by inactivating hydrogen peroxide through the production of α-ketoglutarate[66]. Although GDH screening of stool specimens for the diagnosis of CDI diagnosis is common[67], its value is limited to being a preliminary test, since both toxigenic and non-toxigenic strains produce GDH[68]. GDH is highly conserved among *C. difficile*

strains; no differences in reactivity were found among 168 *C. difficile* isolates belonging to 77 different ribotypes using three different assays carried out by two different groups[68]. As the GDH assay may be positive for *C. difficile* strains that do not produce toxins, a positive GDH assay needs a confirmatory test (CCNA, EIA, a molecular test, or TC)[23,35,67].

GDH has been incorporated, as an initial test, into multistep algorithms (ACG guidelines). Recently, there has been a tendency towards a two-step algorithm, where in the absence of detection of toxin by EIA, clinical evaluation should also be applied to determine true CDI or colonization. This has recently been recommended by ESCMID[69].

The C. Diff Quik Chek Complete assay is a rapid membrane EIA that combines the detection of both GDH and the toxins A and B[70]. With TC as a reference, the C. Diff Quik Chek Complete had a sensitivity of 63.6% and a specificity of 98%[70]. Both assays proved to be highly sensitive (range: 97.6% to 100%) and accurate (88% true positive or true negative). Discrepant results were resolved with the Xpert C. diff assay[71]. The algorithm allowed to rule out *C. difficile* without additional tests when GDH is negative and to confirm CDI when both GDH and toxin A/B results are positive. Another GDH test, the automated Vidas *C. difficile* GDH assay (bioMérieux, Marcy l'Etoile, France), has a 95% agreement with the C. Diff Quik Chek assay (Quik Chek-60, Techlab, United States)[72]. In case of discrepant results, molecular tests are recommended by various authors, because CCNA and TC are expensive and time-consuming[35,73]. A two-step algorithm (step 1, GDH and toxin detection by EIA; step 2, loop-mediated isothermal amplification) yielded a sensitivity of 81%, a specificity and positive predictive value (PPV) of 100%, and a negative predictive value (NPV) of 96%[74]. Even though the sensitivity was lower than in other studies, the PPVs and NPVs supported the practice of reporting the specimen as positive without further testing. A more complex algorithm increased the CDI detection rate from 8% to 19%; initial positive GDH testing (VIDAS *C. difficile*; bioMérieux) was confirmed by toxin testing (VIDAS *C. difficile* Toxin A&B; bioMérieux). In case a toxin EIA did not confirm a positive GDH, additional tests [nucleic acid amplification test (NAAT), CCNA, or TC] were executed on positive GDH samples. A positive confirmatory test is considered CDI-positive sample[75].

Apart from providing improved diagnostic performance, multistep algorithms are the most cost effective for various reasons: (1) because they avoid unnecessary or inadequate treatment and its consequences[76]; and (2) an initial screening with a cheap GDH test allows rapid identification of negative samples, limiting the use of more expensive NAAT tests to only those samples that were positive for GDH[77].

Detection of toxins by enzyme immunoassays

One of the first strategies for the diagnosis of CDI was the detection of toxins with a specific EIA. Several EIA-based kits are commercially available in different formats, such as: lateral flow immunoassay, also known as immunochromatography or strip tests; and solid-phase assays, for example micro-wells[15]. EIA-based confirmation of CDI is practical, fast, and cheap, but it is also one of the least consistent methods (sensitivity range: 63% to 94%, specificity range: 75% to 100%)[23]. According to the SHEA and IDSA guidelines, toxin detection by EIA is less sensitive than detection by CCNA; thus, EIA should not be used alone for the diagnosis of CDI to avoid a false-negative result[23]. A two-step or three-step algorithm that combines GDH screening with EIA improves CDI diagnosis[23,24].

The inconsistent sensitivity of EIAs may be due to several factors, such as: antigenic variation among the toxins of different circulating strains, inadequate storage and transportation of samples, freeze-thaw cycles, and inter-laboratory technical variance, among others[15]. Some of the early developed EIAs accounted only for the detection of toxin A; however, there are reports of strains that do not produce toxin A[78-80]. Also, some assays detect both toxins A and B plus the detection of GDH[81].

Using TC and CCNA as reference, six commercially available EIAs and three lateral-flow assays for the detection of *C. difficile* toxins A and B have been compared. The sensitivities ranged from 60% to 81.6%, whereas the specificities ranged from 91.4% to 99.4%. PPVs and NPVs were diverse and depended on whether the samples originated from a low-prevalence environment (community) or a high-prevalence environment (hospital setting). Though PPVs were low for both settings, the PPV was higher in the high-prevalence setting, independent from the gold standard chosen as a reference. NPVs were high for both settings (above 95%), regardless of the reference chosen[82]. In a two-step algorithm, the initial screening with GDH detection (C.Diff Chek-60, TechLab/Wampole) yielded a sensitivity of 93.4% and a specificity of 96.6% (reference assay: TC). Next, only positive specimens were confirmed with a rapid toxin A/B assay (Tox A/B Quik Chek, TechLab/Wampole, Blacksburg, VA), which yielded a specificity and a PPV of 97.1% and 96.5%, respectively. Compared to TC, the sensitivity of the EIA-based toxin assay was low (52.9%)[83].

Nucleic acid amplification tests

Nowadays, many infections are diagnosed *via* molecular tests. The new generation of NAATs amplify and detect pathogen-specific DNA or RNA sequences. Advantages of NAATs include high sensitivity, high specificity, and speed. Because no viable cells are needed, sampling, handling, transportation, and storage aspects are simplified. Furthermore, no culture is required. The role of NAAT in the diagnosis process for CDI may be supportive[84], part of a two or three-step algorithm according to ESCMID guidelines (Figure 1)[23], or as a stand-alone test in cases of documented

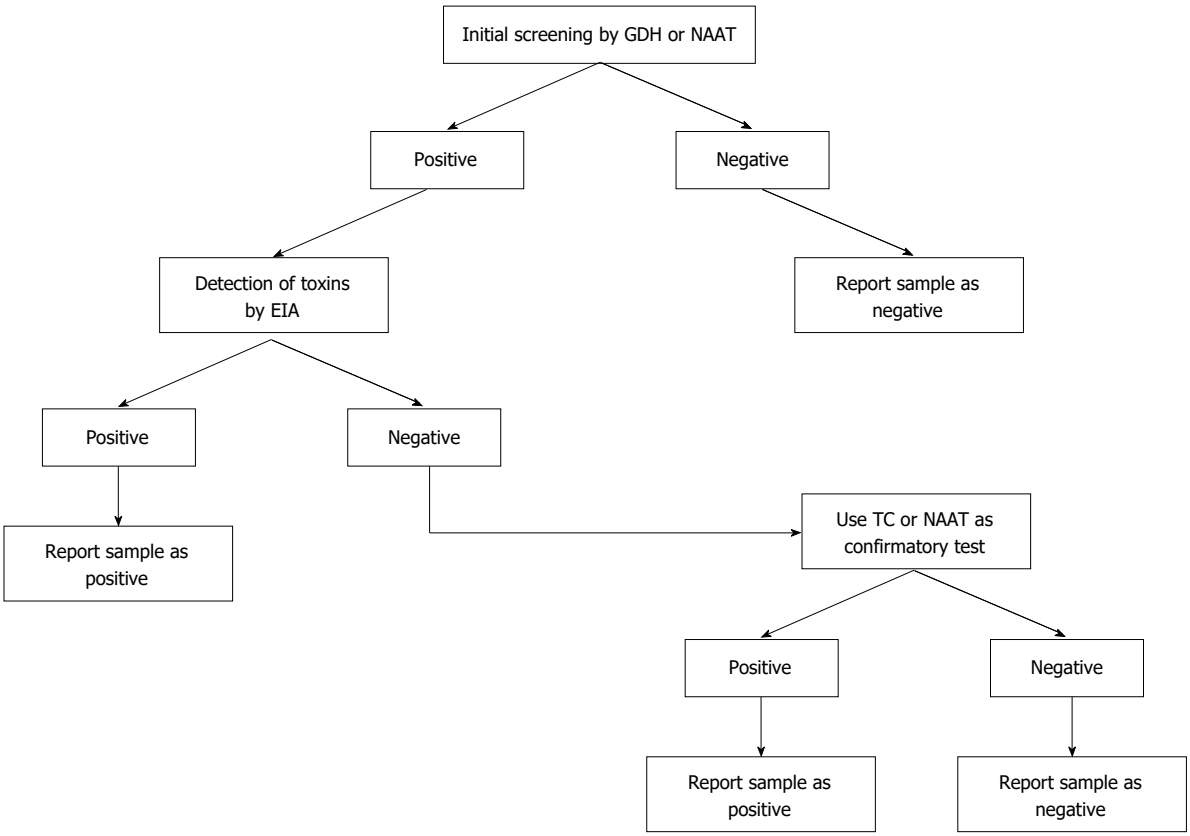

Figure 1 Multistep algorithm for the laboratory diagnosis of *Clostridium difficile* infection based on the European Society of Clinical Microbiology and Infectious Diseases guidance document. GDH: Glutamate dehydrogenase; EIA: Enzyme immunoassay; NAAT: Nucleic acid amplification test; TC: Toxigenic culture.

Table 2 Sensitivity and specificity of nucleic acid amplification test assays for the detection of *Clostridium difficile*

Assay	Sensitivity	Specificity	Ref.
Cepheid Xpert *C. difficile*	90%-100%	92.9%-98.6%	[87,89,91]
IMDx *C. difficile* for Abbott *m*2000 Assay	62.1%-92.8%	99.4%-100%	[92,93]
BD Max Cdiff Assay	81.6%-96.9%	95%-95.8%	[92,93]
Portrait Toxigenic *C. difficile* Assay	98.2%	92.8%	[95]
Quidel Lyra Direct *C. difficile* Assay	82.1%-85.7%	96.9%-98.3%	[96]
Verigene *C. difficile* nucleic acid test	95.2%-98.7%	87.5%-99.4%	[97,128]
Simplexa *C. difficile* Universal Direct real time PCR	87%-98%	100%	[99,128]
AmpliVue *C. difficile* assay	91%-96%	89%-100%	[99,100]
Illumigene *C. difficile* assay	93.3%-100%	95.1%-100%	[95,101]
BD GeneOhm Cdiff assay	89.6%-97.4%	96.7%-98.5%	[95,103]
ProGastro Cd assay	77.93%-100%	93.4%-99.2%	[103,104]

C. difficile: *Clostridium difficile*.

diarrhea[24].

Despite the evident advantages, important issues to be considered before introducing CDI NAATs in the clinical laboratory are: the requirement of trained personnel and higher costs, and the probability of false-positive results because of the high sensitivity of the test and the detection of strains that do not produce toxins. Especially in stool samples from diarrhea cases due to other pathogens, false-positive results may not be recognized as such because of concomitant asymptomatic *C. difficile* carriage. Stool samples that were positive for both NAAT and toxin test had more bacteria and toxins than stool samples that were only NAAT positive[85]. The latter is an argument against the use of NAAT as a stand-alone test.

Single-plex NAATs: The FDA of the United States of America has cleared a set of commercially available single-plex NAATs. Table 2 summarizes the sensitivities and specificities of these tests, which often is over 90% or even close to 100%; these values depend on the test that was used as a reference test, which was

often TC.

Cepheid Xpert® *C. difficile* and Xpert *C. difficile*/Epi: The Cepheid Xpert® *C. difficile* (Sunnyvale, CA) is a real-time PCR assay that detects the *tcdB* gene and thus allows CDI diagnosis, but without strain specification[86]. The multiplex RT-PCR assay Xpert *C. difficile*/Epi not only detects the *tcdB* gene but also the binary toxin genes (*cdtA* and *cdtB*), and a single-nucleotide deletion of the *tcdC*. Therefore, the Xpert *C. difficile*/Epi identifies ribotype 027[87]. Furthermore, Xpert *C. difficile*/Epi detects ribotype 033, a strain of veterinary importance that has been reported in cattle, veal calves, piglets, horses, and soil from various geographical locations worldwide[88]. The binary toxins of ribotype 033 were correctly amplified in all isolates (n = 52) included in a study. However, since ribotype 033 lacks the *tcdA* and *tcdB* genes, the GeneXpert Dx system reports that the sample is negative for CDI, and it is necessary to access the raw data in the instrument to obtain the amplification information[88]. Using TC as a reference, the sensitivity of the Cepheid Xpert *C. difficile*/Epi assay was 90%; its specificity, 92.9%; its PPV 71.4%; and its NPV 97.9%[89].

The introduction of the Cepheid Xpert *C. difficile* assay in a tertiary hospital significantly increased the rate of detection of toxigenic *C. difficile* from 4.7% to 9.9%. The increase was mainly due to cases that yielded indeterminate results with the C. Diff Quik Chek, but were positive with the Xpert *C. difficile* assay[90].

When the performance of the Xpert *C. difficile* Epi assay as a confirmatory test was compared to TC in a two-step algorithm with a GDH assay as a first step, there was a moderate agreement (kappa score 0.48) of 72.6%. The GDH-TC algorithm had a sensitivity of 57% and specificity of 97%, whereas the sensitivity of the GDH-Xpert algorithm was 100% and its specificity 97%. Furthermore, 42 out of 45 stool samples that were ribotype 027 positive were confirmed by PCR-ribotyping and sequencing, indicating a good epidemic value of the assay[91].

IMDx *C. difficile* for Abbott *m*2000 assay: The IMDx *C. difficile* for Abbott *m*2000 assay (IMDx) is a real-time PCR that detects not only *C. difficile tcdA* and *tcdB* genes, but also the rare variant strains rare toxin A^+B^- and toxin B variant (*tcdBv*) gene, which occurs in A^-B^+ strains. Lysis of the sample, target amplification, and detection are performed in the *m*2000 RealTime System (Abbott Laboratories, Abbott Park, IL)[92].

In a prospective analysis of 111 stool specimens and a retrospective analysis of 88 stool samples, in which the IMDx was compared to another FDA-cleared NAAT (the GeneOhm Cdiff Assay), the sensitivity was strain dependent: 100% for NAP1 strains and 90.3% for non-NAP1 strain with a limit of detection of 2250 colony-forming units[92]. However, when IMDx and 2 other molecular assays were compared to TC, IMDx had the lowest sensitivity (62.1%) and the highest specificity (99.4%)[93].

BD Max Cdiff assay: The BD Max Cdiff assay detects and amplifies the *tcdB* gene in a real-time PCR assay performed on the BD Max System (BD Diagnostics, Sparks, MD). After the addition of the sample, this hands-free platform combines DNA extraction and amplification. To extract genetic material a 10-μL loop is immersed in the specimen; next, the loop content is dispersed in BD Max Sample Buffer. The DNA extraction utilizes magnetic beads, which are eluted before a lyophilized amplification mix is added. The results are reported only as positive or negative for *C. difficile*[92].

Compared to the GeneOhm Cdiff Assay, BD Max Cdiff Assay had a sensitivity of 96.9% and a specificity of 95%[92]. In the same study, ribotyping was assessed, but there were no significant differences between the sensitivities and specificities of different ribotypes[94]. When the BD Max Cdiff Assay and 2 other molecular assays were compared to TC, the BD Max Cdiff Assay had a sensitivity of 81.6% and a specificity of 95.8%[93].

Portrait Toxigenic *C. difficile* assay: The Portrait Toxigenic *C. difficile* assay (Great Basin, West Valley City, UT) amplifies a 78-nucleotide fragment of the *tcdB* gene. The assay uses isothermal helicase-dependent amplification, followed by detection with an immobilized capture probe on a sliding array. Each reaction mixture contains three controls: a sample processing control, a hybridization control, and a detection control. Results for the specimen are reported only when the detection criteria for all controls are met[95].

A multicenter evaluation that included 49 stool specimens from 4 clinical sites compared the Portrait Toxigenic *C. difficile* Assay to TC on the same specimens. The sensitivity ranged from 92.9% to 100%, with an overall sensitivity of 98.2%. The specificity ranged from 88.9% to 96.9%, with an overall specificity of 92.8%[95]. When comparing Portrait Toxigenic *C. difficile* Assay results with those from other FDA-cleared tests, the concordance were as follows: 97.5% with Xpert *C. difficile*, 96.4% with GeneOhm Cdiff, and 93.8% with Illumigene *C. difficile*[95].

Quidel Lyra Direct *C. difficile* assay: The Quidel Lyra Direct *C. difficile* assay (Quidel, San Diego, CA) uses qualitative real-time PCR technology. Specimens are tested in a standard TaqMan real-time PCR assay utilizing primers/probes that detect but do not distinguish the *tcdA* and *tcdB* genes. The Lyra assay may be performed on any of three open-platform, real-time thermocyclers: SmartCycler II (Cepheid, Sunnyvale, CA), ABI 7500 Fast DX (Applied Biosystems, Carlsbad, CA), and ABI QuantStudio DX (Applied Biosystems, Carlsbad, CA). The Lyra assay has a running time

of about 3 h[96]. Depending on the platform used, the sensitivity and specificity may differ; the ABI 7500 instrument is the most sensitive and the ABI QuantStudio DX is the most specific. The overall sensitivity is 85.7% and the overall specificity is 98.3% when compared to toxigenic culture[96].

Verigene *C. difficile* nucleic acid test: The Verigene *C. difficile* nucleic acid test is a multiplex qualitative assay that amplifies DNA by PCR in a nanoparticle-based microarray that targets the *tcdA* and *tcdB* genes and differentiates the hypervirulent strain 027/NAP1/BI *via* the binary toxin genes and the base pair deletion at position 117 in the regulator *tcdC* gene[97]. The Verigene system contains two modules: the Verigene Processor SP performs nucleic acid extraction, PCR amplification, and hybridization of amplicons; The Verigene Reader scans the test cartridge, realizes the optical analysis, and generates the results[97].

When compared to TC, the Verigene assay has sensitivity of 98.7% and a specificity of 87.5%[97]. With regard to strain typing, the assay assigns correctly 89.7% of hypervirulent strains compared with ribotyping[97]. When compared to fecal culture as a reference method, the Verigene *C. difficile* nucleic acid test was sensitive (96.7%), specific (97.4%), and accurate (97.1%)[98].

Simplexa *C. difficile* Universal Direct real-time PCR: This assay uses fluorescent bifunctional probes-primers to amplify a *tcdB* fragment. Samples in Tris-EDTA buffer are heat-treated; the lysate is used directly to perform the test. The system can accommodate a maximum of 94 samples and has an assay time of 91 min. When compared to another FDA-cleared assay, the Meridian Illumigene Asssay, the Simplexa *C. difficile* Assay had a sensitivity of 98% and a specificity of 100%; the concordance between the two systems was 98.7%[99].

AmpliVue *C. difficile* assay: The AmpliVue *C. difficile* assay uses helicase-dependent, isothermal amplification of a highly conserved 83-bp fragment of the *tcdA* gene. The assay includes a disposable detection device that allows for visual evaluation of amplification results. The AmpliVue system can perform a maximum of 24 assays, and has a total running time of 73 min. The AmpliVue assay had a sensitivity of 96% and specificity of 100% when compared to the FDA-cleared assay Meridian Illumigene Assay[99]. Compared to TC, the sensitivity and specificity were 91% and 89%, respectively[100].

Illumigene *C. difficile* assay: The Illumigene *C. difficile* assay uses loop-mediated isothermal DNA amplification technology to target a partial DNA conserved region of *tcdA* common to A+B+ and A-B- strains. The total time of analysis is 68 min and the maximum number of samples per run is 10[99]. When compared to TC, the sensitivity and specificity of the Illumigene assay were 100%[101].

BD GeneOhm Cdiff assay: The BD GeneOhm Cdiff assay (BD Diagnostics, San Diego, CA) amplifies the *tcdB* gene from stool samples with *C. difficile,* which is detected and analyzed with a SmartCycler instrument (Cepheid, Sunnyvale, CA)[102]. The performance of the BD GeneOhm Cdiff PCR assay has been compared to the Tox A/B II ELISA and a two-step method composed of the C. Diff Chek-60 GDH antigen assay followed by cytotoxin neutralization on 105 true positive samples. The detection rate was 66.7% for the Tox A/B II ELISA assay, 82.9% for the 2-step method, and 91.4% for the BD GeneOhm Cdiff PCR assay. The overall concordance between the BD GeneOhm Cdiff PCR assay and the Tox A/B II ELISA was 91.3%, while the concordance between the BD GeneOhm Cdiff and the two-step method was 93%. The BD GeneOhm Cdiff PCR and the two-step algorithm had similar performance but were more sensitive than Tox A/B II. Compared to the two-step algorithm, BD GeneOhm Cdiff PCR is faster but almost five times more expensive; BD GeneOhm Cdiff PCR is also six times more expensive than the Tox A/B assay[102]. When compared to TC, BD GeneOhm Cdiff PCR has a sensitivity of 89.6% and a specificity of 96.7%[103].

ProGastro Cd assay: ProGastro Cd assay (Prodesse, Waukesha, WI) is a Taqman real-time PCR assay that detects the *tcdB* gene. Amplification is performed on the Cepheid SmartCycler II (Sunnyvale, CA). Stool samples are processed to obtain genetic material using the NucliSENS easy MAG platform (bioMérieux, Inc., Durham, NC)[104].

There was a 95.7% agreement between TC and the ProGastro Cd assay. When comparted to TC, the ProGastro Cd assay had a sensitivity that ranged from 77.3% to 100% and a specificity between 99.2% and 93.4%[103,104]. A two-step algorithm, with the GDH-based C. Diff Quik Chek Complete as step 1 and the ProGastro Cd assay as step 2, yielded an estimated sensitivity of 97.9% and a specificity of 95.4%.

Multiplex platforms: There are two types of multiplex platforms: the first type includes the so-called "syndromic" platforms*, i.e.,* a platform to detect pathogens associated with a particular symptom. In this case, a variety of syndromic platforms test for the main causative agents of diarrhea, independently of the kind of microorganism (bacteria, viruses or protozoa). The second type are pathogen class specific multiplex molecular assays[105].

Currently, there are two FDA-cleared syndromic multiplex assays that include the dectection of *C. difficile*: Luminex xTAG GPP (Luminex Molecular

Table 3 Summary of non Food and Drug Administration-approved multiplex assays that detect *Clostridium difficile*

Assay	Company	Pathogens detected	Technology	Ref.
Gastrofinder Smart 17 Fast	PathoFinder	9 bacteria, 4 viruses and 4 parasites	Multiplex Real-time PCR	[129]
EasyScreen Enteric assays	Genetic Signature	7 bacteria, 8 viruses and 5 parasites	3base Technology	[130]
RIDA®GENE	R-BioPharma AG	11 bacteria, 4 viruses and 4 parasites	Multiplex Real-time PCR	[131]
FTD® Bacterial Gastroenteritis	Fast-Track Diagnostics	9 bacteria, 5 viruses and 3 parasites	Multiplex Real-time PCR	[132]
CLART EnteroBac panel	Genomica	19 bacteria	Low-density microarray	[133]
Faecal Bacteria	AusDiagnostics	8 bacteria, 4 viruses and 3 parasites	Multiplex Tandem PCR technology	[134]
Seeplex Diarrhea ACE	Seegene	10 bacteria and 4 viruses	Dual priming oligonucleotide technology	[135]

Diagnostics Inc., Toronto, Canada) and BioFire FilmArray GI Panel (BioFire Diagnostics, Salt Lake City, UT)[106]. In addition, there is a non-FDA cleared, but "Conformité Européene" (CE) marked multiplex assay, the Gastrofinder Smart 17 Fast (PathoFinder, Maastricht, The Netherlands)[105]. There are six additional commercially available platforms that include *C. difficile* among their targets, but none of these platforms are FDA cleared or CE marked[105].

Luminex xTAG pathogen panel: This assay simultaneously detects *Salmonella* sp., *Shigella* sp., Shiga toxin-producing *E. coli* (STEC) stx1/stx2, *Vibrio cholerae*, *Yersinia enterocolitica*, *C. difficile* toxin A/B, *Campylobacter* sp., *E. coli* O157, Enterotoxigenic *E. coli* (ETEC) LT/ST, adenovirus 40/41, rotavirus A, norovirus GI/GII, *Giardia lamblia*, *Cryptosporidium* sp. and *Entamoeba histolytica*[107]. The assay performs a multiplex reverse transcriptase PCR, using tagged and biotinylated primers. Amplicons are detected by hybridization to the pathogen-specific complementary antitag sequence coupled to specific beads and binding of the streptavidin-phycoerythrin reporter to the biotinylated primers[106,107].

The performance of the Luminex xTAG panel was evaluated with 185 stool samples from 176 patients. In 11% of the samples, multiple pathogens, including ETEC, *G. lamblia*, norovirus, *Shigella* sp., *Campylobacter* sp., *Salmonella* sp., adenovirus, and *C. difficile,* were detected[107]. There was a 100% sensitivity, specificity, PPV, and NPV. Another study evaluated the assay with a total of 254 clinical specimens[108]. Depending on the target organism, the sensitivity ranged from 90% to 100% with an overall sensitivity of 94.5% and a specificity of 99.1%. With respect to *C. difficile*, the sensitivity, specificity, PPV, and NPV were 91%, 100%, 100%, and 99%, respectively.

In a site-specific clinical evaluation, the Luminex xTAG Gastrointestinal assay showed a sensitivity of 98.7% and a specificity of 99.8%. *C. difficile*, *Salmonella* spp., and *Cryptosporidium* spp. accounted for 67% of the targets detected. Specifically, *C. difficile* was detected in 23 samples, all of which were confirmed with the Cepheid Xpert *C. difficile* assay. Furthermore, the multiplex detection led to savings in the hospital ward as compared to traditional methods[109].

FilmArray GI panel: The FilmArray uses nested multiplex PCR that is executed in two stages. First, the FilmArray performs a reverse transcription PCR. Next the diluted products are combined with a fluorescent double-stranded DNA-binding dye. This solution is aliquoted into an array with wells that contain primers to amplify internal sequences of the first product, so that in each well an individually nested PCR is performed. Results are obtained after fluorescence analysis[106,110].

The assay detects *Campylobacter* (*C. jejuni*, *C. coli* and *C. upsaliensis*), *C. difficile*, *Plesiomonas shigelloides*, *Salmonella*, *Y. enterocolitica*, *Vibrio* (*V. parahaemolyticus*, *V. vulnificus,* and *V.cholerae*), *E. coli* O157, Enteroaggregative *E. coli*, Enteropathogenic *E. coli*, ETEC, STEC, *Shigella*/Enteroinvasive *E. coli*, Adenovirus F 40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, Sapovirus (Ⅰ, Ⅱ, Ⅳ, and Ⅴ), *Cryptosporidium*, *Cyclospora cayetanensis*, *E. histolytica* and *G. lamblia*. For the identification of *C. difficile*, the FilmArray GI Panel targets both *tcdA* and *tcdB*[111].

In a study that evaluated the BioFire FilmArray GI Panel, *C. difficile* was the most frequently detected pathogen (83 out of 378 samples, 22%). The sensitivity of the BioFire FilmArray Gastrointestinal Panel to detect *C. difficile* was 95% and the specificity 99% using the Illumigene as a reference. In 91 episodes for which specific testing for *C. difficile* was ordered, 42 episodes (46%) were *C. difficile* positive according to standard testing and 40 (44%) according to the FilmArray GI Panel in 40 (44%)[112].

In a multicenter evaluation, the BioFire FilmArray GI Panel detected *C. difficile* in 204 out of 832 positive samples, contrary to 165 positive samples detected by the comparator (PCR of toxin A and B genes), resulting in a positive percent agreement of 98.8% and negative percent agreement of 97.1%[111].

Non-FDA approved assays

There are seven non FDA-approved assays on the market that include *C. difficile* among their targets (Table 3). The majority of these assays, employ real-time PCR technology. To our knowledge, only three of them have been evaluated regarding the sensitivity and specificity at detection *C. difficile*.

The RIDA® GENE CD PCR assay (R-Biopharm AG, Darmstadt, Germany), when compared to TC as a reference, had a sensitivity of 98.1%, a specificity

of 100%, a PPV of 100%, and a NPV of 98.1%[113]. Ylisiurua *et al*[113] found that the RIDA® GENE CD PCR assay outperforms competing molecular *C. difficile* assays, such as GeneOhm™ Cdiff assay (Becton Dickinson) and Xpert® C. difficile test (Cepheid).

The EasyScreen Enteric Bacterial Detection Kit is a multiplex assay with calculated analytical sensitivities that range from 2.5 to 12.5 copies of targeted pathogen, free of cross-reactivity to non-target microorganisms. For *C. difficile*, the calculated sensitivity was 100% and the specificity 81.2%. The EasyScreen assay correctly identified all *C. difficile*-containing samples (12 out of 18), including the ribotype 027 and 078 strains (1 of each)[114].

The Seeplex® Diarrhea ACE is another multiplex molecular assay that was compared to BD GeneOhm, with TC as a reference. There was a positivity rate of 35.4% (86/243). The concordance rate between the BD GeneOhm and Seeplex® Diarrhea ACE assay was 96% (234/243) with no significant differences between them. The sensitivity, specificity, PPV, and the NPV of the Seeplex® Diarrhea ACE assay were 90.0% (63/70), 97.1% (168/173), 92.6% (38/43), and 96.0% (168/175), respectively[115].

BIOMARKERS

CDI is accompanied by intestinal inflammation. Inflammation biomarkers include cytokines, calprotectin, and fecal lactoferrin[116]. These biomarkers are not disease specific, but may be indicators of severity[117]. For example, fecal lactoferrin has been evaluated in both infectious diarrhea and inflammatory bowel disease. Fecal lactoferrin, blood biomarkers, white blood cell count and low serum albumin level, were significantly associated with severe CDI and stool toxin[116]. Furthermore, age, Charlson co-morbidity index, intensive care treatment, increased peripheral white blood cell count, elevated lactoferrin, decreased albumin, and elevated creatinine were significantly associated with death within 100 d of CDI diagnosis[118].

Calprotectin is a protein found in the cytoplasm of neutrophils and can be detected in stool in cases of intestinal inflammation, such as in inflammatory bowel disease and infectious diarrhea[117,119]. A large proportion of individuals with nosocomial diarrhea (diagnosed by PCR) have elevated levels of calprotectin[120]. High fecal levels of calprotectin have been associated with *C. difficile* strain 027[121] and complicated/recurrent CDI in a cohort of older adults[119]. Though elevated stool calprotectin has a low sensitivity as a diagnostic test (38.5%), its specificity for complicate or recurrent CDI was 91.9%, and thus provides valuable information for adequate treatment decision-making[119].

So far, there is no specific biomarker that detects CDI or any other pathogenic agent of clinically significant diarrhea. Anikst *et al*[122] suggests the implementation of measures to avoid unnecessary testing for *C. difficile* in order to diminish CDI overdiagnosis. The identification of such a biomarker, will be helpful to improve CDI diagnosis.

CONCLUSION

C. difficile has a worldwide distribution; its toxigenic strains are responsible for CDI. Despite increasing knowledge on risk factors that favor CDI and measures to reduce propagation, there is an increase in the prevalence of CDI in many countries[123-127]. One of the challenges at managing of CDI is the initial diagnosis of the disease. To date, there is no single test that accurately and rapidly diagnoses CDI. Multistep testing is recommended for a diagnosis with acceptable sensitivity and specificity. The inclusion of NAATs in the diagnostic algorithm combines high sensitivity with a short turnaround time. However, test results should be interpreted with caution and should consider clinical suspicion, the presence of risk factors, and a correct interpretation of test results. A better understanding of the pathogenesis of *C. difficile* will help both physicians and laboratories to develop the best strategy to overcome current issues with CDI diagnosis.

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ORIGINAL ARTICLE

Basic Study

hsa-mir-183 is frequently methylated and related to poor survival in human hepatocellular carcinoma

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Abstract

AIM

To screen clinically relevant microRNAs (miRNAs) silenced by DNA methylation in human hepatocellular carcinoma (HCC).

METHODS

Knockdown of DNA methyltransferases (DNMTs) using siRNAs and miRNA profiling in HCC cell lines were performed to identify DNA hypermethylation-mediated miRNA downregulation. Confirmation using individual quantitative real-time PCR (qRT-PCR) assays was then

performed followed by DNA methylation quantification at the promoter of the miRNA genes. Quantification of DNA methylation and miRNA expression was then performed in primary HCC tumor samples and related with clinicopathological variables.

RESULTS

miRNA profiling after DNMT knockdown in HCC cell lines revealed upregulation of miR-23, miR-25 and miR-183. After qRT-PCR confirmation and CpG island methylation quantification of these miRNAs in cell lines, further analysis in primary HCC specimens showed that *hsa-miR-183* is hypermethylated in 30% of HCC (n = 40). Expression of mature miR-183 showed an inverse correlation with DNA methylation levels. In HCC cells, *DNMT* knockdown and 5-aza-2′-deoxycytidine treatment reduced methylation and stimulated expression of miR-183. In HCC patients, hypermethylation at *hsa-miR-183* promoter significantly correlates with poor survival (log-rank test P = 0.03). DNA methylation analysis in healthy liver, benign liver tumors (hepatocellular adenoma and focal nodular hyperplasia) and their corresponding adjacent tissues showed absence of hypermethylation supporting the notion that aberrant methylation at *hsa-miR-183* is specific for the malignant transformation of hepatocytes.

CONCLUSION

Our data indicate that hypermethylation of *hsa-miR-183* is a frequent event in HCC and potentially useful as a novel surrogate diagnostic and prognostic marker.

Key words: *hsa-miR-183*; DNA methylation; *DNMT* knockdown; MicroRNA microarray

Core tip: A comprehensive screening using microRNA microarray in hepatocellular carcinoma (HCC) cells after *DNMT1*-, *DNMT3A*-, and/or *DNMT3B*-knockdown revealed upregulation of miR-23, miR-25, and miR-183. Using primary HCC tumor tissues, we confirmed frequent DNA hypermethylation at the *hsa-miR-183* promoter. Hypermethylation of *hsa-miR-183* was not found in benign liver tumors, adjacent tumor tissues as well as healthy livers and significantly correlated with poor prognosis. Therefore it represents a potential novel diagnostic and prognostic marker in HCC.

Anwar SL, Krech T, Hasemeier B, Schipper E, Schweitzer N, Vogel A, Kreipe H, Buurman R, Skawran B, Lehmann U. *hsa-miR-183* is frequenty methylated and related to poor survival in human hepatocellular carcinoma. *World J Gastroenterol* 2017; 23(9): 1568-1575 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1568.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1568

INTRODUCTION

A class of small non-coding RNAs with the ability to negatively regulate gene expression, named microRNA (miRNA), has been revealed to greatly contribute to tumor development and progression since the last decade[1]. Rather than completely blocking expression of the target genes, miRNAs act specifically in post-transcriptional modulation by fine-tuning of gene expression[2]. Differential expression of miRNAs has been described in almost all tumor types thus also eliciting new opportunities to utilize miRNA as a potential diagnostic or prognostic marker. Depending on their functions, miRNAs are either up- or down-regulated in cancer. After the first report revealing inactivation of miRNA genes by DNA methylation[3], epigenetically mediated silencing of various miRNAs have been reported across different types of human cancers, including human hepatocellular carcinoma[4,5].

DNA methylation aberrations have been shown to contribute to the early steps of malignant transformation[6]. Both miRNA expression and DNA methylation patterns display tissue specificity. Therefore, cancer profiling using those two features have successfully discriminated tumor from healthy tissues and also classified subtypes of tumors with a notable clinical relevance[7].

HCC is among the top five major types of cancer with more than 750000 new cases diagnosed each year and accounts for the third highest cancer mortality rate worldwide[8,9]. However, knowledge about epigenetic aberrations of miRNA genes in HCC is still surprisingly limited. Only few studies have addressed DNA methylation-mediated miRNA silencing in HCC[5,10]. Using *in silico* and *in vitro* screening, our previous study has shown frequent DNA methylation aberrations in intergenic miRNA genes and its potential value for specific detection and as a new marker for poor survival in HCC[4]. In the human genome, around 60% miRNAs are intergenic, located far away from any other known genes. The remaining 40% of miRNAs are located within introns of protein-encoding genes. Transcription of these intragenic miRNAs is likely to be regulated in the same direction as the host genes[11]. Some intragenic miRNAs have also been reported to be epigenetically inactivated in HCC[12].

To complement our previous studies, we used a comprehensive experimental screening approach to identify yet unknown hypermethylation mediated miRNA silencing in HCC. Specific DNA methyltransferase (*DNMT*) knockdown followed by miRNA expression profiling in HCC cell lines was performed. Upregulated miRNAs after *DNMT* knockdown were selected according to the presence of CpG islands in the 5 kb distance to avoid indirect and unspecific effects of DNMT knockdown. Following this strategy we identified *hsa-miR-183* as a new target of aberrant

Table 1 Clinopathological variables of hepatocellular carcinoma, hepatocellular adenoma, and focal nodular hyperplasia patients involved in this study

	HCC (n = 40)	HCA (n = 10)	FNH (n = 5)
Age			
< 50	12	10	4
> 50	28	0	1
Sex			
Male	33	1	2
Female	7	9	3
Etiology			
HBV	8		
HCV	4		
No infection	28		
Tumor differentiation			
Good	15		
Moderate	17		
Poor	8		
Tumor size			
< 5 cm	20	4	4
> 5 cm	20	6	1
Stage			
Ⅰ	5		
Ⅱ	11		
Ⅲ	16		
Ⅳ	8		
Number of nodules			
Unilocular	14	9	4
Multilocular	26	1	1
Cirrhosis			
With cirrhosis	32		
Without cirrhosis	8		
Survival			
< 3 yr	18		
> 3 yr	17		
No information	2		
Diagnosed < 2 yr ago	3		

HCC: Hepatocellular carcinoma; HCA: Hepatocellular adenoma; FNH: Focal nodular hyperplasia; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

DNA methylation in primary HCC specimens.

MATERIALS AND METHODS

Patient samples and cell lines

Primary tumor specimens were collected at the time of surgery from 40 patients with HCCs, 10 with hepatocellular adenoma (HCA), and 5 with focal nodular hyperplasia (FNH) operated at the Hannover Medical School Germany following a protocol approved by the local ethics committee ("Ethik-Kommission der Medizinischen Hochschule Hannover", head: Prof. Dr. Tröger). After snap froze in liquid nitrogen, the primary tissues were stored at -80 ℃ until the time of analysis. Clinical specimens were assessed by two independent pathologists following universally accepted criteria for HCC, HCA, and FNH. Morphologic classification of liver tumors and grading of hepatocellular carcinoma were performed according to recommendations as previously described by Lehmann *et al*[13] and Schlageter *et al*[14]. Tumor cell content was verified by the pathologists to be at least 70% using HE staining from the reference sections of the snap frozen samples. Basic clinicopathological variables of the patient samples are summarized in Table 1. Seven HCC cell lines (HLE, HLF, HuH7, HepG2, Hep3B, SNU182, and SNU387) and two immortalized hepatocyte lines (THLE-2 and THLE-3) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, United States) and grown in conditions recommended by ATCC. Identity of all cell lines was validated using short tandem repeat (STR) profiling following the DSMZ's protocol. All experiments using cell lines were performed at subconfluent cellular density allowing exponential growth.

DNMT1, *DNMT3A*, and *DNMT3B* knockdown

DNMT gene knockdown experiments in HLE cells were performed with pre-designed pools of four siRNA targeting *DNMT1, DNMT3A, DNMT3B* (ON-TARGET plus and siGENOME SMARTpool siRNAs, Dharmacon/Thermo Scientific, London, United Kingdom) following the manufacturer's protocol. In brief, 2×10^4 cells in 500 μL complete medium were seeded in 24-well plate simultaneously with 100 μL of a previously prepared mixture containing 50 or 100 nmol/L siRNA/well, Lipofectamine™ RNAiMAX (Invitrogen, Darmstadt, Germany), and Opti-MEM (Gibco-Invitrogen, Darmstadt, Germany) following recommendations from the manufacture. After 24 h medium containing transfection reagent was replaced by new medium and repeated transfection was performed after 48 h from this point onward. After 3 sequential transfections and re-plating into 12- and 6-well plates, cells were harvested for RNA, DNA, and protein extractions. Two scramble siRNAs (AllStars Negative Control siRNA, Qiagen, Hilden, Germany and Riboxx® control-N1, Riboxx, Dresden, Germany) were included in the experiments as negative controls.

Western blot analysis

Fifteen μg of protein lysate was separated in 10% pre-cast SDS-polyacrylamide gels (Bio-RAD, Munich, Germany) and transferred onto Hybrid-P polyvinylidene difluoride membrane (Amersham Biosciences, Freiburg, Germany). Antibodies used were mouse monoclonal anti-DNMT1 antibody (IMG-261A clone 60B1220.1, Imgenex, San Diego, CA, United States), mouse monoclonal anti-β-actin antibody (ab6276 clone AC-15, Abcam, United Kingdom), and anti-mouse secondary antibody HRP (R1253HRP, Acris, Herford, Germany).

DNA and RNA extraction

Extraction of high molecular weight DNA from the fresh-snap-frozen primary specimens was performed by digestion with proteinase K (Merck, Darmstadt, Germany) followed by phenol/chloroform procedure (ROTI® Carl Roth GmbH, Karlsruhe, Germany) following standard procedures. Total RNA was extracted

Table 2 List of primers used in this study

Pyrosequencing	Forward	Reverse	Ta (℃)	$MgCl_2$ (mmol/L)	Sequencing
miR-23 cluster	TTTAAGTYGTGTGAAATTATGTGGTAG	ATAAACACCRAAAAACRAATCCA	60	2.5	TGGTAGTTTATGGTTGTGAG
miR-25 cluster	GTGYGGGTTAATYGGATAAGG	AAAAACCCRACRCCTACACTAC	60	2.5	GYGGGTTTAGAATGAGT
miR-183	GTTATTAATAGGAATGGGGTAG	AAACRACTCTCAACCTCCC	60	1.5	GAATGGGGTAGTTGAGGG

using TRIZOL™ reagent (Invitrogen, Darmstadt, Germany). For miRNA profiling, RNA was extracted using miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer.

miRNA profiling

Comprehensive miRNA expression profiling after *DNMT* knockdown was performed with Agilent's high-performance miRNA Microarray Platform (Release 19.0, 8x60K) using its set of optimized reagents and hardware following the supplier's instruction as previously described in detail[15].

Reverse transcription and quantitative real-time PCR

Reverse transcription of mature miRNA species was performed using 10 ng RNA per each reaction using High Capacity cDNA Reverse Transcription Kit and TaqMan® miRNA assays (Applied Biosystem, Darmstadt, Germany) following the manufacturer's recommendation (ID numbers of the TaqMan® assays: 002270 for hsa-miR-183-3p and 002269 for hsa-miR-183-5p. For normalization, RNU48 and U6 were used as reference transcripts. Quantitative real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystem, Darmstadt, Germany) on ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, United States). For mRNA quantification after DNMTs-knockdowns, RNA (1 μg) was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's recommendation. Hs00154749_m1 for DNMT1, Hs01027166_m1 for DNMT3A, Hs00171876_m1 for DNMT3B and two references Hs00939627_m1 (GUSB) and Hs02758991_g1 (GAPDH) were used for mRNA expression analysis after DNMTs-knockdown. Expression of miRNAs in primary tumor samples was displayed as fold change after normalized with the paired adjacent liver tissues.

Bisulfite conversion and methylation analysis

For DNA methylation analysis, genomic DNA was treated with sodium bisulfite using EZ DNA Methylation Kit™ (Zymo Research, HiSS Diagnostics, Freiburg, Germany) according to the manufacturer's recommended protocol. For each PCR amplification, approximately 25 ng of the bisulfite modified DNA was used. DNA methylation analysis was performed with pyrosequencing as initially described[4] using newly designed primers as available at Table 2. DNA methylation level for a given sample was presented as the mean of all CpG dinucleotide methylation values from two independent pyrosequencing runs. The software Pyro-Q-CpG™ (Qiagen, Hilden, Germany) was used for analyzing DNA methylation levels of each individual CpG dinucleotides. "Hypermethylated" was then defined as methylation value above mean of the adjacent liver tissue plus two times the standard deviation ($Mean_{adj.} + 2 \times StD$).

DAC treatment in HCC cell lines

DAC treatment in HLF, HuH7, and HepG2 cells in final concentration 100 nmol/L for 5 d was performed as initially described[4].

Statistical analysis

Clustering analysis of the miRNA profiling after DNMT knockdown was performed using Qlucore Omics Explorer v2.2 (Qlucore, Lund, Sweden). For statistical analysis, GraphPad Prism (version 5.01 for Windows, La Jolla, CA, United States) was used. The Mann-Whitney-*U* test was utilized to compare continuous variables and χ^2 test for relationships between categorical variables. To compare survival of HCC patients, Kaplan-Meier curve and long-rank (Mantel-Cox) test were used. For those comparisons, $P < 0.05$ was considered as statistically significant.

RESULTS

Upregulated miRNAs after DNMT knockdown

Upon *DNMT-1*, *-3A*, and *-3B* knockdowns (Supplementary Figure 1), several miRNAs were shown to be upregulated (Figure 1A). To sort out upregulation of miRNAs directly affected by demethylation due to the *DNMT* knockdown, we further analyzed presence of CpG island association (within 5 kb) at the miRNA gene promoters. After *DNMT* knockdown, *hsa-miR-23* cluster, *hsa-mir-25* cluster, and *hsa-mir-183* cluster were shown to be upregulated by using miRNA profiling (Figure 1A). To show direct effects of DNMT knockdowns to alterations of DNA methylation and miRNA expression, we used HLE cells upon *DNMT1* knockdown and measured methylation of *hsa-miR-183* promoter and expression miR-183. *DNMT1* knockdown in HLE cells led to decreased methylation at *hsa-miR-183* promoter ($P = 0.0015$) accompanied by elevated miR-183 expression ($P = 0.04$) (Figure 1B).

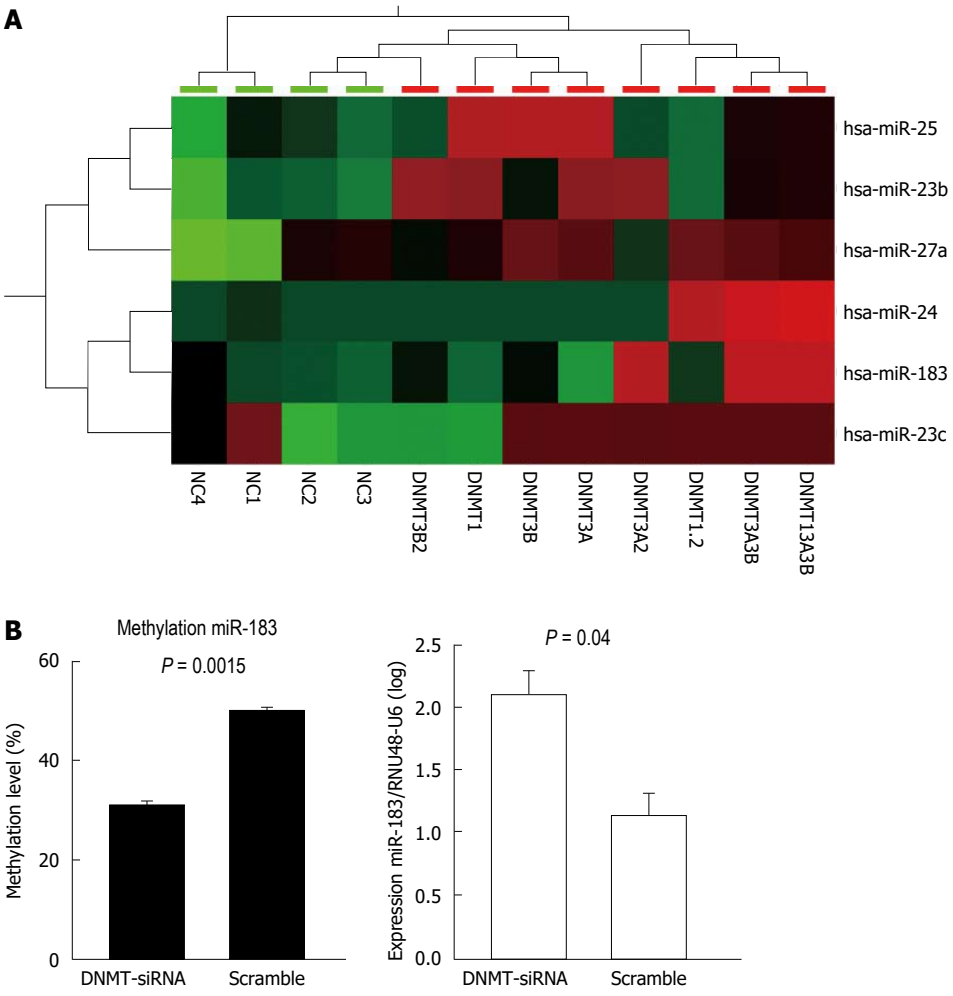

Figure 1 Upregulated microRNAs after DNA methyltransferases knockdown (A); and Methylation and expression changes of *hsa-miR-183* after DNA methyltransferases knockdown (B). A: Cluster analysis after single and combined *DNMT* knockdown using siRNAs (as indicated in the lower panel) shows upregulation of selected microRNAs (right panel). High, intermediate, and low microRNA expression levels in the heatmap are indicated with red, black, and green, respectively. B: Upon DNMT1 knockdown in HLE cell lines, DNA methylation at the promoter of miR-183 decreased significantly (right panel, $P = 0.0015$) accompanied by elevated miR-183 expression (left panel, $P = 0.04$). NC = negative control siRNA and DNMT (1, 3A, 3B) = DNA methyl transferase 1, -3A, -3B siRNA treated samples. DNMT: DNA methyltransferase.

Differential DNA methylation in primary HCC specimens

Methylation analysis was performed at promoters of the upregulated miRNAs after *DNMT* knockdown. First, we performed DNA methylation analysis at the promoter of *hsa-miR-23* cluster, *hsa-mir-25* cluster, and *hsa-mir-183* cluster in 7 HCC cell lines (HLE, HLF, Huh7, HepG2, SNU182, and SNU387) and 2 healthy adult liver epithelial lines (THLE2 and THLE3). Differential DNA methylation was not observed at the promoter of *hsa-mir-25* cluster among HCC cell lines and healthy adult liver epithelial lines indicating that the upregulation after *DNMT* knockdown is likely due to indirect effects. Therefore, further DNA methylation analysis at the promoter of *hsa-mir-25* cluster in clinical samples (primary HCC, HCA, FNH tissue samples) was not performed. At the promoter of *hsa-miR-23* cluster, although differential methylation was shown in cell lines, we observed constant hypermethylation both in HCC and the adjacent liver tissue (Supplementary Figure 2). We observed differential methylation at *hsa-mir-183* transcriptional start site in HCC compared to the corresponding adjacent liver tissues. Hypermethylation was demonstrated in 30% of HCC tumor samples ($n = 40$) (Figure 2).

Deregulated miRNA expression in relation with DNA methylation status

In order to evaluate the functional relevance of differential DNA methylation at *hsa-mir-183* locus, we performed expression analysis of mature miR-183 in primary HCC specimens. MiR-183 was frequently downregulated in HCC and its expression negatively correlated with the methylation status (Figure 3).

DNA methylation analysis in benign liver tumors and healthy liver tissues

We further analyzed DNA methylation patterns at *hsa-mir-183* in benign liver tumor samples. Altogether 10 cases of HCA, 5 cases of FNH, their corresponding adjacent liver tissues, as well as 5 healthy liver tissues

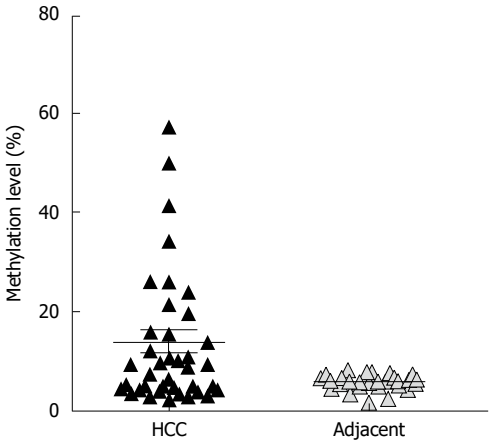

Figure 2 Differential DNA methylation at *hsa-mir-183* promoter in primary hepatocellular carcinoma specimens compared to the adjacent liver tissues. High-resolution quantitative DNA methylation analysis using pyrosequencing shows 30% of HCC samples with hypermethylation. "Hypermethylation" was defined as described in Materials and Methods. HCC: Hepatocellular carcinoma.

were analyzed. Aberrant methylation at *hsa-mir-183* was not found in benign liver tumors and healthy liver tissues (Supplementary Figure 3). This indicates that aberrant methylation at *hsa-mir-183* might occur only during malignant transformation of hepatocytes.

Impact on HCC survival

HCC patients with hypermethylation at *hsa-miR-183* locus had a significant shorter survival (log-rank test, $P = 0.03$; Figure 4). Age is not significantly different between HCC patients with and without hypermethylation indicating that the gain of methylation at *hsa-miR-183* is related to biological course of the disease not merely due to an increase in age. Hypermethylated samples are found more frequently in late HCC although this difference is not statistically significant.

DISCUSSION

The discovery of miRNA has brought significant improvement in the understanding of regulation of gene expression and highlighted the emerging of a novel class of regulatory molecules in gene transcription[7]. The ability of miRNAs to control gene expression post-transcriptionally reveals a complex and interrelated regulatory network of gene expression. Later, it has been also shown that miRNAs are functionally regulated by epigenetic mechanisms including DNA methylation and histone modifications[3,4,11]. Aberrant DNA methylation and histone modification have been suggested to be an important causal factor for deregulation of miRNA expression, especially in cancer cells.

Liver tumors comprise a range of benign, premalignant, and malignant lesions that differ in histology, etiology, clinical management, and outcome. Aberrations of both DNA methylation and miRNA expression are very specific depending on developmental stage,

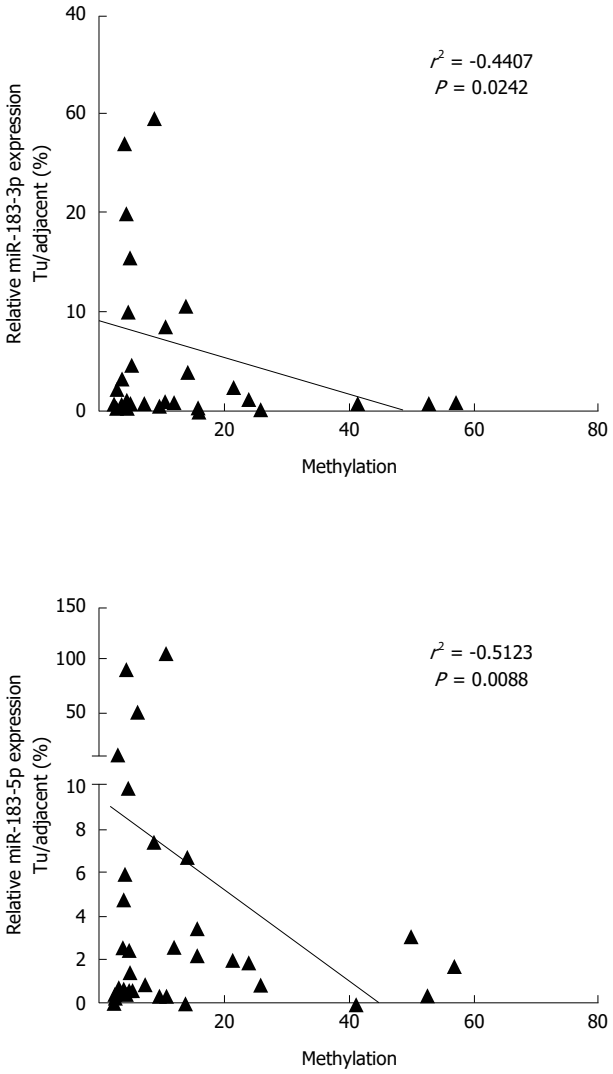

Figure 3 DNA hypermethylation leads to repression of *hsas-miR-183* transcription. Shown are scatter plots demonstrating negative correlation between DNA methylation levels and miR-183 expression case by case.

cell differentiation, and tissue types[5,16,17]. Therefore, using combination of DNA methylation and miRNA expression as clinical relevant markers is potentially of practical relevance in some complex and heterogeneous diseases including liver cancer[5]. Using an *in silico* screen, our previous study has revealed that aberrant DNA methylation of miRNA genes was a biological event detected only in malignant liver cells and tissues but not in adjacent tumor tissues, benign liver lesions, and in hepatocyte lines[4]. Aberrant DNA methylation at miRNA loci does not occurs at random but appears as highly organized event during the course of hepatocarcinogenesis. Therefore, DNA methylation at miRNA genes has a potential as diagnostic or prognostic marker as well as for guiding alternative adjuvant targeted therapy in HCC because some miRNAs regulated by DNA methylation play a role in modulating therapeutic responses in HCC[5,18,19].

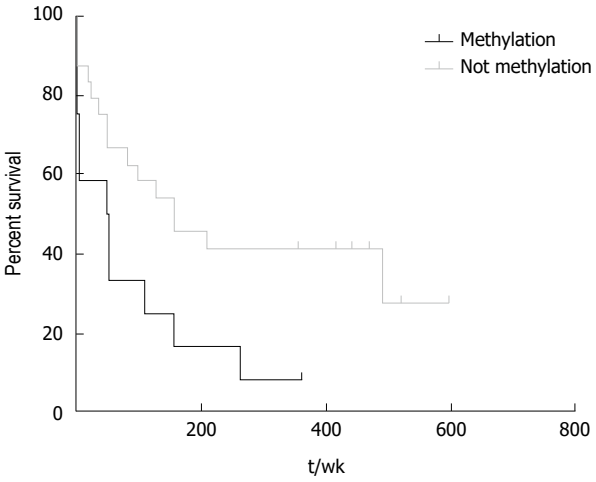

Figure 4 Hypermethylation at *hsa-miR-183* significantly correlates with worse survival in hepatocellular carcinoma. The Kaplan-Meier plot shows comparison of HCC patient survival between those with hypermethylated and non-methylated *hsa-miR-183* locus (log-rank test $P = 0.03$, median survivals are 49.5 and 150 wk for patients with hypermethylation and without increased methylation at *hsa-miR-183* locus respectively). HCC: Hepatocellular carcinoma.

In addition to the *in silico* screen previously done by us[4], we performed an experimental screen by using siRNA mediated *DNMT* knockdown to induce re-expression of miRNA expression silenced by DNA methylation. Our experimental screen revealed that *hsa-mir-183* is upregulated after DNMT knockdown. Using primary HCC specimens, aberrant DNA methylation is detected in up to 30% of total samples. *DNMT1* knockdown leads to significant reduction of DNA methylation at the promoter and increased expression of mature miR-183. Downregulation of miR-183 has been previously described in several cancers such as lung cancer[20], breast cancer[21], or osteosarcoma[22]. Upregulation of miR-183 has also been reported in liver cancer precursors including cirrhotic and pre-malignant lesions[23]. The discrepancy of miR-183 expression in cirrhosis, premalignant liver lesions, and liver cancer might reflect the dynamic evolution during carcinogenesis since miRNAs modulate several mRNAs and transcription factors. Mature miR-183 expression in HCC can be activated by β-catenin through elevated synthesis of polycistronic transcripts of the *hsa-miR-182-96-182* cluster[24]. Therefore, in the presence of *CTNNB1* mutations, levels of miR-183 expression might not show the expected downregulation in HCC due to complex interactions of this genetic alteration with promoter DNA methylation. In addition, upregulation of miR-183 was not correlated with survival of HCC patients[25] indicating that expression alone is not a useful prognostic marker in HCC.

Our present study showed frequent aberrant DNA methylation at the promoter of *hsa-miR-183*. *Hsa-mir-183* itself is an intergenic miRNA and located at chromosome 7 in a cluster together with *hsa-miR-96* and *hsa-miR-182*. The putative transcriptional start site is predicted at 5207 bp upstream of *hsa-mir-183* within a CpG island adjacent to this cluster (CpG 351). The pyrosequencing assay for the detection of DNA methylation used in this study was developed for this CpG island. Aberrant methylation of *hsa-miR-183* was absent in benign liver lesions (HCAs, FNHs), adjacent liver tissues (from HCC, HCA, and FNH), as well as healthy liver tissues. This indicates that hypermethylation of *hsa-miR-183* is a potential diagnostic marker to differentiate malignant liver tumor from benign lesions especially in cases where relying on histopathology alone might be difficult, for example in distinguishing HCA from well-differentiated HCC. Although involving limited numbers of HCC patients, our present study also shows that aberrant DNA methylation at *hsa-miR-183* promoter is associated with poor HCC outcome. Hypermethylation of *hsa-miR-183* might not be able to replace other prognostic factors commonly used to predict HCC survival, but offer an additional relevant marker to determine HCC prognosis.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality with limited treatment options, mainly due to late detection of disease. Epigenetic alterations like DNA methylation are now recognized as important contributors to the development and progression of malignancy in humans. However, our knowledge about epigenetic alterations in HCC and their clinical relevance is still limited.

Research frontiers

Determining the clinical relevance of DNA methylation aberrations in microRNA genes and exploring the potential as new biomarker of this epigenetic aberration.

Innovations and breakthroughs

Comprehensive microRNA expression screen after DNA methyltransferase (DNMT) knockdown, avoiding the unspecific side effects of "classical epigenetic screens" using nucleotide analogues like azacytidine or aza-deoxycytidine as DNMT inhibitors.

Applications

hsa-miR-183 gene methylation as new prognostic marker for the clinical management of HCC patients.

Peer-review

In this manuscript, Anwar *et al*, described the status of miR-183 methylation and related to poor survival in human HCC. In general, this is an interesting manuscript, explaining epigenetic regulation of miR-183 in HCC and its potential role as a novel surrogate and prognostic marker.

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ORIGINAL ARTICLE

Basic Study

Porcine model characterizing various parameters assessing the outcome after acetaminophen intoxication induced acute liver failure

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Author contributions: Thiel K, Klingert W, Klingert K, Morgalla MH, Schuhmann MU, Leckie P, Sharifi Y, Schenk M and Thiel C performed the animal experiments; Thiel K, Davies NA, Jalan R, Schenk M and Thiel C designed the study and coordinated the study group; Morgalla MH and Schuhmann MU contributed to the neurosurgical part of the experimental setting; Klingert K and Grasshoff C provided advice for the standardized intensive care therapy; Thiel K, Davies NA, Grasshoff C, Schenk M and Thiel C drafted the manuscript; Klingert W, Peter A and Königsrainer A helped to draft the manuscript and participated in its design; Klingert W and Schenk M carried out the biochemical and statistical analysis; Peter A supervised all biochemical and laboratory measurements; all authors read and approved the final manuscript.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at christian.thiel@med.uni-tuebingen.de.

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Abstract

AIM

To investigate the changes of hemodynamic and laboratory parameters during the course of acute liver failure following acetaminophen overdose.

METHODS

Eight pigs underwent a midline laparotomy following jejunal catheter placement for further acetaminophen intoxication and positioning of a portal vein Doppler flow-probe. Acute liver failure was realized by intra-jejunal acetaminophen administration in six animals, two animals were sham operated. All animals were invasively monitored and received standardized intensive care support throughout the study. Portal blood flow, hemodynamic and ventilation parameters were continuously recorded. Laboratory parameters were analysed every eight hours. Liver biopsies were sampled every 24 h following intoxication and upon autopsy.

RESULTS

Acute liver failure (ALF) occurred after 28 ± 5 h resulted in multiple organ failure and death despite maximal support after further 21 ± 1 h (study end). Portal blood flow (baseline 1100 ± 156 mL/min) increased to a maximum flow of 1873 ± 175 mL/min at manifestation of ALF, which was significantly elevated ($P < 0.01$). Immediately after peaking, portal flow declined rapidly to 283 ± 135 mL/min at study end. Thrombocyte values (baseline 307 × 10^3/μL ± 34 × 10^3/μL) of intoxicated animals declined slowly to values of 145 × 10^3/μL ± 46 × 10^3/μL when liver failure occurred. Subsequent appearance of severe thrombocytopenia in liver failure resulted in values of 11 × 10^3/μL ± 3 × 10^3/μL preceding fatality within few hours which was significant ($P > 0.01$).

CONCLUSION

Declining portal blood flow and subsequent severe thrombocytopenia after acetaminophen intoxication precede fatality in a porcine acute liver failure model.

Key words: Acetaminophen intoxication; Acute liver failure; Portal blood flow; Thrombocytopenia; Animal model; Porcine model

Core tip: It still remains difficult to predict the outcome in patients with acute liver failure (ALF). Therefore we aimed to investigate the clinical course of portal blood flow (PBF) and changes in thrombocyte count in a porcine model of acetaminophen induced ALF. At manifestation of ALF, PBF increased maximally, followed by a rapidly decline until death due to multiple organ failure. In addition, thrombocytes values declined slowly at the onset of ALF. In the early ALF course, a second decline appeared 8 h after ALF escalating to a more severe thrombocytopenia after 16 h in ALF preceding fatality within few hours.

Thiel K, Klingert W, Klingert K, Morgalla MH, Schuhmann MU, Leckie P, Sharifi Y, Davies NA, Jalan R, Peter A, Grasshoff C, Königsrainer A, Schenk M, Thiel C. Porcine model characterizing various parameters assessing the outcome after acetaminophen intoxication induced acute liver failure. *World J Gastroenterol* 2017; 23(9): 1576-1585 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1576.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1576

INTRODUCTION

Acetaminophen (Paracetamol®) can cause severe hepatic injury if taken in large amounts either unintentionally or with suicidal intent[1]. Currently it represents the most common cause of acute liver failure (ALF) in both United States and United Kingdom with a trend to increasing incidence[2]. Various prognostic models have been established to predict the outcome of patients suffering from ALF[3-9]. The most widely accepted criteria are those defined by King's College Hospital[10], which include predominantly biochemical parameters including arterial pH, prothrombin time and serum creatinine to identify patients in need of liver transplantation. Unfortunately, a variety of biochemical parameters in ALF are deranged representing indices of altered biochemical pathways or epiphenomena of liver necrosis. Furthermore the King's criteria was questioned by Bailey *et al*[11] in a systematic review and meta-analysis of established prognostic scores. The authors concluded that all criteria presently available are insensitive and may miss patients requiring emergency liver transplantation. The crucial question as to whether the liver regenerates spontaneously, or that liver transplantation will be unavoidable remains the most important challenge for the attending physician. To address this problem, animal models analysing the pathophysiologic alterations in the course of ALF provide an opportunity to improve therapeutic strategies and investigate new prognostic parameters. The requirements for parameters of interest are availability, feasibility and robust assurance in diagnosis. Although acetaminophen toxicity has been extensively studied in rat[12,13] and mouse models[14,15], the precise pathogenic mechanisms of hepatocyte damage which are potentially reversible are still poorly understood. Recently, large models of

acetaminophen intoxication have been established in porcine models[16,17] which are more appropriate to represent the human liver physiology and the clinical course of acetaminophen poisoning. These models are also of sufficient size to allow use of a representative human intensive care setting, which permits testing of novel therapeutic interventions.

Based on observations of a preliminary animal study of acetaminophen intoxication in which a distinctive pattern of portal blood flow (PBF) and alterations in thrombocyte count were measured[17], the following animal study was aimed to characterize the clinical course of PBF and changes in thrombocyte count during the development, onset and further course of acetaminophen induced ALF.

MATERIALS AND METHODS

Animal model

After approval by the institutional review board for animal experiments, all experiments were performed according to the international principles governing research on animals and under the supervision of a veterinarian, who set the guidelines for minimizing suffering.

The study was performed in eight female German landrace pigs weighing 38 ± 2 kg. Premedication, anaesthesia, intensive care medication and algorithms for standardized intensive care management have been previously reported in detail[18].

In brief, intramuscular premedication consisted of atropine 0.1% (0.05 mg/kg), ketamine (14 mg/kg), azaperone (2 mg/kg) and midazolam (0.5 mg/kg). Pigs were orally intubated and ventilated with a pressure controlled ventilation modus. Continuous intravenous anesthesia consisted of ketamine (15 mg/kg per hour), fentanyl (0.02 mg/kg per hour) and midazolam (0.9 mg/kg per hour).

Surgical procedures

The jugular and femoral veins as well as the femoral artery were instrumented to measure central venous pressure (CVP) and mean arterial pressure (MAP). A probe was inserted in the frontal brain parenchyma to record intracranial pressure. Following a median laparotomy the portal vein was separated and a 10-15 mm peri-vascular Doppler flow-probe (Medi-Stim, Oslo, Norway) was placed without restricting the portal vein diameter, and fixed to periportal tissue. PBF data were recorded electronically by a CM-2000 Doppler Flowmeter (Medi-Stim, Oslo, Norway). A jejunal catheter (Gentle-Flo™, Tyco Healthcare, Tullamore, Ireland) was inserted into the upper jejunum for further acetaminophen administration and a 14F urinary catheter (Ruesch Care, Kernen, Germany) was placed by cystostomy. The abdominal wall was closed with a running suture. Liver biopsies were sampled every 24 h following intoxication and upon autopsy. Clear ascites (500 to 1500 mL) was removed in the intoxication group during liver biopsy procedures which were performed surgically by reopening the abdomen.

Acetaminophen intoxication and ALF

Two pigs (2/8) were sham operated as a control group and received no acetaminophen intoxication. Dosage, administration and acetaminophen plasma level monitoring for a reproducible onset of acute liver failure after intrajejunal acetaminophen intoxication has been previously described in detail[17]. In brief, six pigs (6/8) received an initial enteric acetaminophen bolus of 250 mg/kg body weight *via* the implanted jejunal catheter. Intoxication was continued initially by an enteric maintenance dose of 2000 mg acetaminophen every hour. Acetaminophen plasma levels were recorded every four hours to adapt acetaminophen maintenance dose (1000-3000 mg) to targeted plasma levels between 300-450 mg/L.

The onset of the ALF syndrome was defined by presence of coagulopathy represented by a decline of the prothrombin time (PT) value below 30% at which point acetaminophen intoxication was stopped.

Standardized intensive care management

All pigs (8/8) remained under anaesthesia receiving pressure-controlled ventilation until conclusion of the study. Intensive care medication and algorithms for standardized fluid management which were used to ensure hemodynamic stability have been previously reported in detail[17,18]. Relevant vital parameters as electrocardiogram, heart rate, MAP, CVP, intracranial pressure and body temperature were recorded electronically throughout the experiment (IntelliVue MP50, Philips Medical Systems, Andover MA, United States). Arterial blood gas analysis (ABL 800, Radiometer, Copenhagen, Denmark) including haemoglobin, methaemoglobin, hematocrit, lactate, serum electrolytes, acid base balance and blood glucose levels were monitored hourly and ventilation parameters [12-30 breaths/min, tidal volume 6-12 mL/kg and oxygen concentration (FiO2) 0.3 - 1.0] were adjusted accordingly. Complete blood count, PT, aspartate aminotransferase, creatinine, albumin, bilirubin, ammonia and total plasma protein were measured before, immediately after and every eight hours following acetaminophen intoxication until study end. Norepinephrine, in combination with hydroxyethylstarch 6% (Voluven® HES 130/0.4, Fresenius, Bad Homburg, Germany) and sodium chloride solution 0.9% were used to ensure hemodynamic stability. After the onset of ALF, four fresh-frozen plasma units (300 mL/unit) were given within 24 h to avoid spontaneous bleeding complications which were observed in pilot studies. Packed erythrocyte units (300 mL/unit) were given if haemoglobin levels decreased below 6 g/dL. Blood glucose levels were maintained > 100 mg/dL with glucose 20% solution. Sodium bicarbonate 8.4% solution was

used to compensate metabolic acidosis. Death was defined by a decline of MAP below 35 mmHg whilst receiving maximal vasopressor support. Sham animals were killed by a single intravenous bolus of 10 mL T 61 (Intervet, Unterscheißheim, Germany) at 48 post surgery.

Continuous venovenous hemofiltration

At ALF, continuous venovenous hemofiltration was administered to all animals of the intoxication group (6/6). Continuous hemofiltration therapy was administered in both sham animals from 24 h (Prismaflex® system. Gambro, Hechingen, Germany) using a TF 1000 PRE SET membrane filter (Gambro Industries, Meyzieu, France). The device system was washed and primed according to the manufacturer's instruction and connected to the right femoral double-lumen catheter. Hemofiltration settings were: mean blood flow rate of 100 mL/min; filtration rate of 35 mL/kg body weight per hour; with fluid withdrawal at 60 mL/h. Unfractionated heparin (250 IU/h, B. Braun Melsungen AG, Germany) was administered to avoid clotting.

Biochemical analysis

All biochemical parameters including acetaminophen plasma level were measured by the certified central laboratories of the Tuebingen University Hospital (Division of Endocrinology, Diabetology, Angiology, Nephrology, Pathobiochemistry and Clinical Chemistry, Department of Internal Medicine, Tuebingen University Hospital, Germany). Arterial albumin, lactate and creatinine (enzymatic) concentrations were determined on the ADVIA 1800 Clinical Chemistry analyzer, ammonia and acetaminophen plasma concentrations were determined on the Dimension RXL Clinical Chemistry analyzer and the ADVIA 2120 Hematology analyzer was used for blood counts (all Siemens Healthcare Diagnostics, Eschborn, Germany). Coagulation tests were performed on the ACL TOP 700 Hemostasis Testing System, (Instrumentation Laboratory, Kirchheim, Germany). Sample analysis was conducted within 1 h of collection at each time point.

Histological examinations

Biopsy specimens of the liver were obtained every 24 h after intoxication and immediately post-mortem, and fixed in used 4% formaldehyde. Sections of the specimen were routinely stained with haematoxylin-eosin. Additionally Ki-67 immunostaining by using alkaline phosphatase staining was performed.

Statistical analysis and calculation

Mean values were compared by Wilcoxon test (JMP® 8.0, SAS Institute, Cary, NC, United States). Values of the laboratory parameters were compared to baseline values by the Wilcoxon matched pairs test. A P value < 0.05 was considered significant. Results are reported as mean ± SE. The statistical methods of this study were reviewed by Martin Schenk, University Hospital Tuebingen.

RESULTS

Manifestation of ALF

All intoxicated animals (n = 6) developed features of ALF within 28 ± 5 h, confirmed by a decrease in PT below 30%. Mortality due to ALF occurred after 21 ± 1 h due to multiple-organ failure (end of the study). The course of the hemodynamic parameters MAP, CVP, the amount of vasopressor support (norepinephrine dosage) required to maintain organ perfusion, is given in Figure 1A-C. Both sham operated animals (n = 2) survived the observation period of 48 h without substantial changes of hemodynamic and laboratory parameters. Significant hemodynamic changes in MAP occurred 8 h after the onset of ALF (P < 0.05; Figure 1A), CVP 16 h after ALF (P < 0.02; Figure 1B) and norepinephrine dosage 16 h after ALF (P < 0.04; Figure 1C). Slightly elevated intracranial pressure baseline values of 19 ± 1 mmHg (n = 8) resulted from the unphysiological supine position of the animals. An elevation due to ALF was noticed after 16 h (19 ± 2 mmHg in sham animals *vs* 30 ± 2 mmHg in intoxicated animals, P < 0.03) and 24 h (17 ± 1 mmHg *vs* 35 ± 5 mmHg) post ALF.

Course of mean PBF

Portal vein flow remained hepatopetal in all animals throughout the experiment. The course of PBF during acetaminophen intoxication, manifestation of ALF and following multiple-organ failure is shown in Figure 1D. The sham operated animals started with a PBF at baseline of 1016 ± 16 mL/min. The PBF remained stable with an overall flow of 825 ± 91 mL/min throughout. At start of acetaminophen administration in the intoxication group (n = 6) baseline PBF was 1100 ± 156 mL/min. Three animals in the acetaminophen group required between 36-40 h to develop ALF, while the remainder achieved ALF within 16-20 h. Within the animals that required a prolonged acetaminophen loading phase the PBF increased only slightly for the initial period but showed a substantial rise 8 h before manifestation of ALF analogous to the course of the more susceptible animals. Maximum portal blood flow was 1873 ± 175 mL/min (n = 6) at the onset of ALF. The elevation in PBF 8 h before and at the onset of ALF was found to be significant compared to the sham animals at 24 h (P < 0.01). Immediately after ALF, PBF started to decline rapidly to a minimal value of 283 ± 135 mL/min (n = 6) at study end.

Course of PT, thrombocytes and selected ALF related laboratory parameters

The course of PT is shown in Figure 2A. PT in all ALF animals declined constantly following acetaminophen

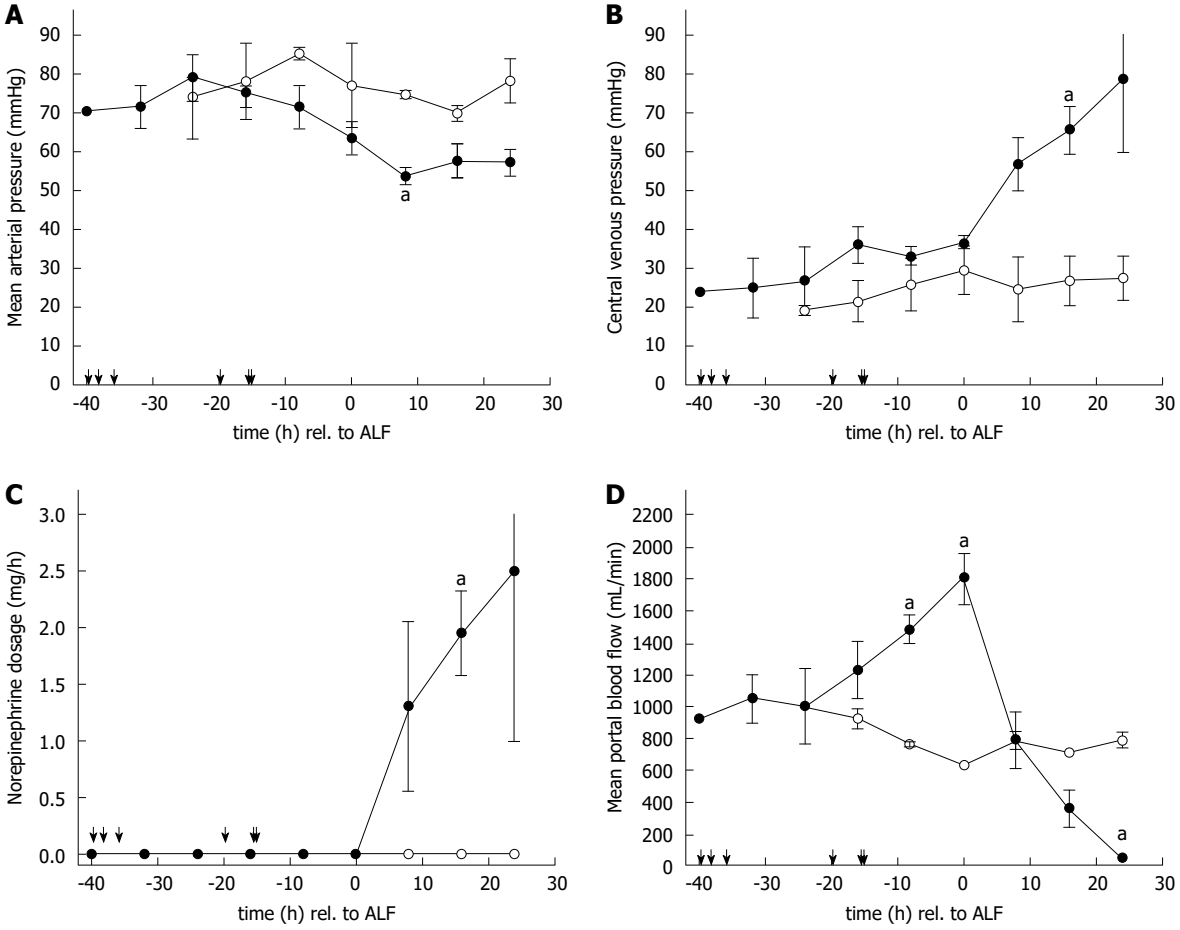

Figure 1 Profile of mean arterial pressure, central venous pressure, norepinephrine dosage and portal blood flow in sham operated and acetaminophen intoxicated animals. The course of mean arterial pressure (A), central venous pressure (B), dosage of norepinephrine (C) and portal blood flow (D) in sham (white line) and acetaminophen intoxicated (black line) animals. All values are given as mean ± SE relative to the onset of acute liver failure (ALF) (in hours). Black arrows on x-axis indicate start of acetaminophen intoxication of individual animals. [a]$P < 0.05$ *vs* sham animals.

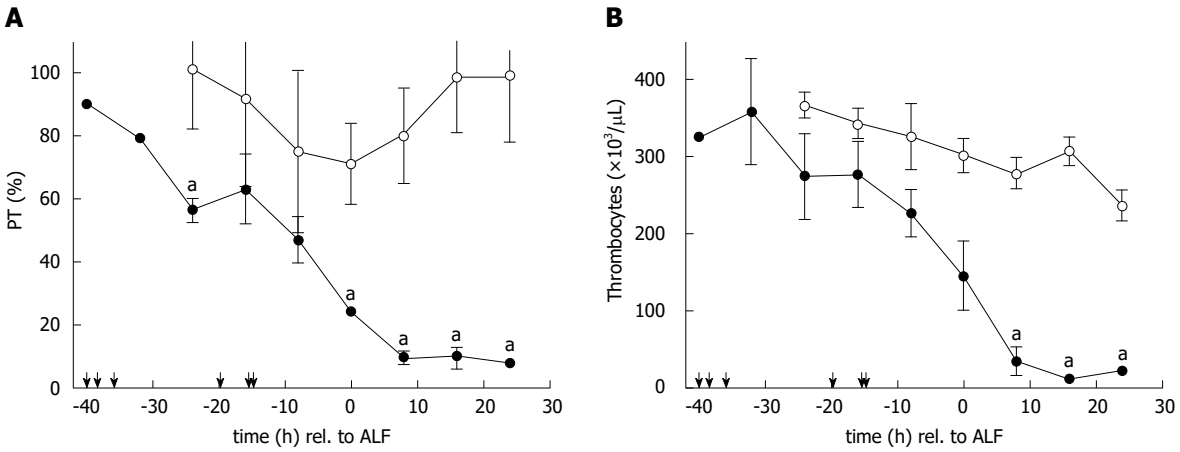

Figure 2 Profile of prothrombin time and thrombocytes in acetaminophen intoxicated and sham animals. The course of prothrombin time (PT) (A) and thrombocytes (B) in sham (white line) and acetaminophen intoxicated (black line) animals. All values are given as mean ± SE relative to the first onset of ALF (in hours). Black arrows on the X-axis indicate start of acetaminophen intoxication of individual animals. [a]$P < 0.05$, *vs* sham animals. ALF: Acute liver failure.

administration. There was a transient decrease in sham animals due to the surgical trauma and volume of fluid resuscitation which later normalised. Baseline thrombocyte values for ALF animals were measured at 307 × 10^3/μL ± 34 × 10^3/μL; and 367 × 10^3/μL ± 18 × 10^3/μL in sham animals. Thrombocyte values for ALF animals declined following acetaminophen to 145 × 10^3/μL ± 46 × 10^3/μL at the onset of ALF.

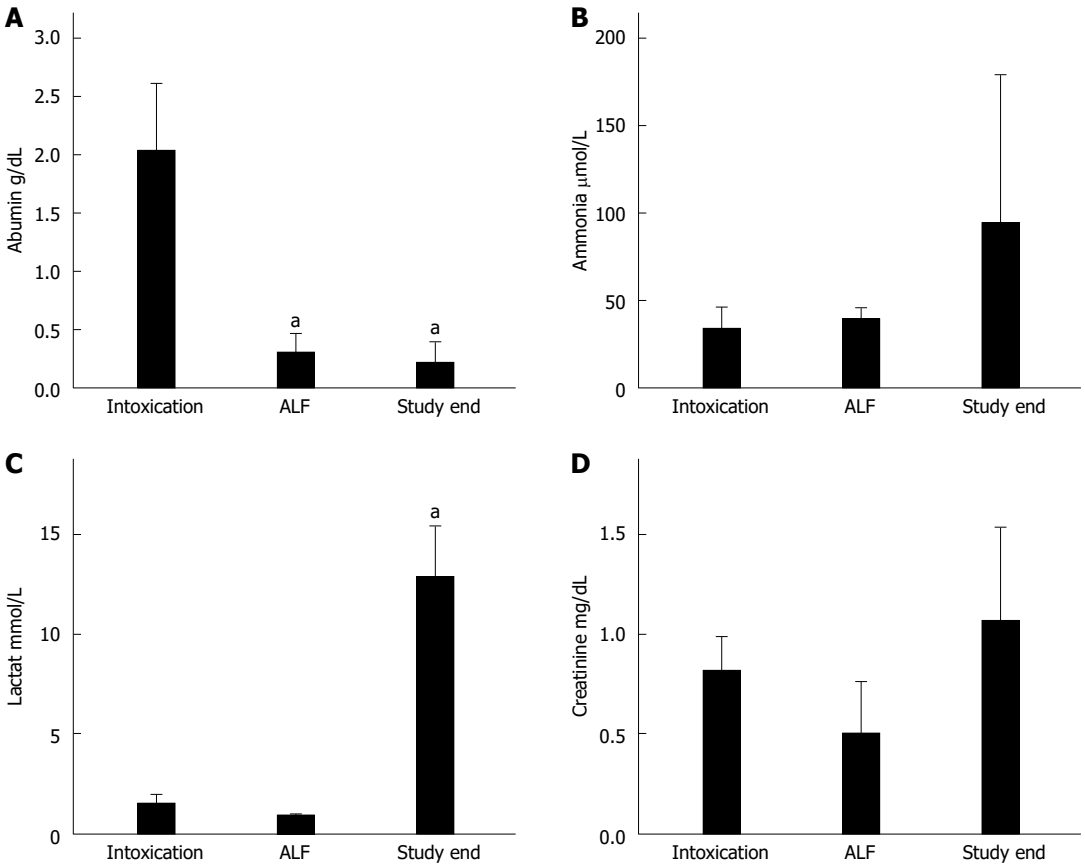

Figure 3 Acute liver failure related laboratory parameters of acetaminophen intoxicated animals. Box plot analysis of the selected laboratory values albumin (A), ammonia (B), lactate (C) and creatinine (D) at the moments: start of intoxication, ALF and study end. All values are given as mean ± SE. $^{a}P < 0.05$, *vs* the moment start of intoxication. ALF: Acute liver failure.

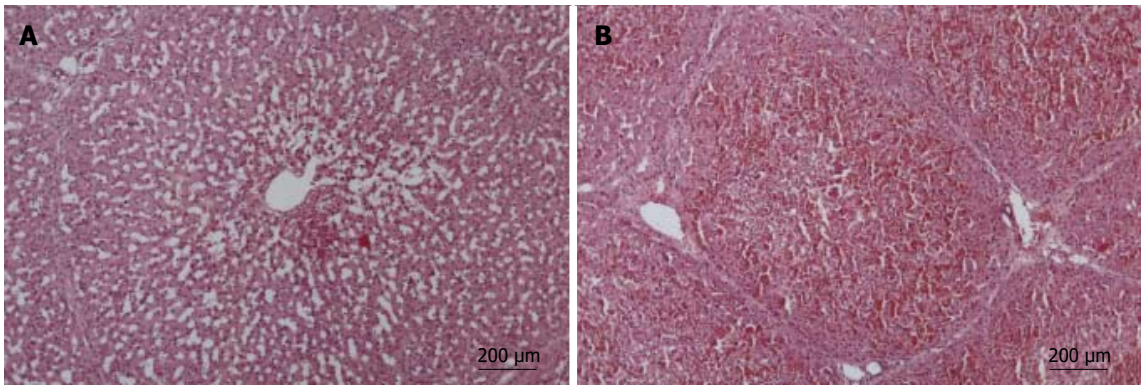

Figure 4 Light microscopic preparations (haematoxylin-eosin staining) of liver biopsies. Biopsies taken 24 h after acetaminophen intoxication show enlarged liver sinus with beginning centrilobular necrosis (A); Exitus biopsy shows progedient centrilobular necrosis (B); Bar indicates 200 µm; objective magnification × 20.

and were further reduced after 8 h post ALF (33 × 10^3/µL ± 18 × 10^3/µL, $P < 0.01$) resulting in severe thrombocytopenia with values of 11 ± 3 × 10^3/µL ($P < 0.01$) after 16 h in ALF preceding death within few hours (Figure 2B). Values of the laboratory parameters are shown in Figure 3. The decline of albumin (baseline 2.0 ± 0.2 g/dL *vs* ALF 0.2 ± 0.1 g/dL *vs* study end 0.2 ± 0.1 g/dL) was statistically significant ($P < 0.05$) at both time-points (Figure 3A). Plasma ammonia levels (baseline 34 ± 5 µmol/L and ALF 39 ± 3 µmol/L) increased to 95 ± 35 µmol/L at study end, though the difference was not found to be significant (Figure 3B). The increase of lactate (baseline 1.5 ± 0.5 mmol/L *vs* ALF 0.9 ± 0.1 mmol/L *vs* study end 12.9 ± 2.6 mmol/L) was significant ($P < 0.05$) at death compared with baseline values (Figure 3C). Creatinine values (baseline 0.8 ± 0.1 mg/dL *vs* ALF 0.5 ± 0.1 mg/dL *vs* study end 1.1 ± 0.2 mg/dL) remained near normal physiological values due to effective continuous venovenous hemofiltration therapy during the course of ALF (Figure

3D).

The increase of plasma aspartate transaminase (baseline 50 ± 9 U/L *vs* ALF 105 ± 28 U/L *vs* study end 237 ± 109 U/L) was significant raised ($P < 0.02$) at death compared with baseline values. Alanine aminotransferase plasma values did not change substantially during the course of ALF (baseline 33 ± 3 U/L *vs* ALF 23 ± 10 U/L *vs* study end 26 ± 7 U/L) and total bilirubin plasma levels remained in the physiological range (baseline 0.1 ± 0.0 mg/dL *vs* ALF 0.1 ± 0.0 mg/dL *vs* study end 0.3 ± 0.1 mg/dL).

Autopsy and histological examinations of liver biopsies

Upon autopsy, massive ascites (2000 to 3000 mL) and a dark-blue necrotic liver was found in all ALF animals. Liver biopsies taken at 24 h showed enlarged liver sinus with early centrilobular necrosis (Figure 4A). Exitus biopsy demonstrated progedient centrilobular necrosis (Figure 4B) in contrast to normal liver architecture in sham operated animals (data not shown). No evidence for increased hepatocyte proliferation (Ki-67 immunostaining) was found in any animals (data not shown). Macroscopically, kidneys were swollen with hemorrhagic infarctions. Histological examination showed acute tubular necrosis and biopsies of brain tissue revealed substantial edema (data not shown).

DISCUSSION

In this present study we sort to characterise the course of PBF and thrombocyte count in order to analyse their prognostic value for predicting a fatal outcome in a porcine model of acetaminophen intoxication. The objective of these studies is to identify the moment at which recovery will be unlikely and emergency liver transplantation remains the ultimate treatment option. The distinctive pattern of PBF and thrombocyte count in combination with worsening hemodynamics were identified as relevant parameters.

The high reproducibility of ALF resulting in 100% mortality due to multiple-organ failure confirmed the results of the our previously published porcine model of acetaminophen intoxication[17]. Within the current experimental setting, continuous venovenous hemofiltration was administrated in addition to standardized intensive care therapy in order to maximally prolong survival in ALF for patients after massive acetaminophen overdose[19]. The use of a continuous renal support device itself, applied to both sham operated animals after 24 h analogous to the onset of ALF in intoxicated animals did not influence the change of mean PBF nor the fall in thrombocytes substantially.

Unexpectedly 3/6 animals needed a longer acetaminophen loading period (36-40 h) for the development of ALF in contrast to other animals (16-20 h). Both subgroups showed different kinetics in PBF changes. The slowly intoxicating animals presented only a slight increase in PBF during the initial intoxication phase followed by a rapid increase within the last 8 h before manifestation of ALF. This phenomenon is likely associated with the animal's individual tolerance towards toxic acetaminophen metabolites. The observation that the rapid increase of PBF within 8 h before ALF in both subgroups suggests that the rapid change in PBF, even before the onset of ALF, is indicative of the extent of toxicity for the animal.

As PBF represents the main component of the liver perfusion and portal vein flow measurement is easily accessible for the attending physician in the clinical setting, we decided to focus on the changes determined in PBF. We abstained from quantifying hepatic artery flow which would increase surgical trauma and which varies considerably following the application of norepinephrine administration in ALF. Although assessment of volume blood flow by Doppler ultrasound is subject to a number of variables, errors can be minimized with careful attention to detail[20]. To evaluate a complete pattern of PBF, continuous recording was obtained by a surgically implanted Doppler flow-probe placed around the portal vein. Portal vein hemodynamics have recently been investigated in liver transplantation[21], in small for size syndrome after extended hepatectomy[22], and in living donor liver transplantation[23] in both clinical and experimental animal studies. The portal vein inflow, within its physiological range, is a stimulus for hepatic regeneration[24,25], but excessive PBF has been shown to be detrimental to the function of the affected liver[26,27]. Several studies investigating the changes of PBF in ALF have been performed in patients by serial Doppler ultrasound examinations[28] or in rats[29] by using a radioactive microsphere technique. They demonstrated a significant increase of PBF in the development and early onset of ALF followed by a steady return to baseline values during liver regeneration. The increase of PBF was clearly identified as a consequence of elevated cardiac output similar to the hemodynamic changes seen in hyperdynamic septic shock. Although the MAP during ALF could be stabilized by intensive care support, a constant decline of PBF was observed in the post ALF period. Norepinephrine which was required for hemodynamic stabilisation in ALF in our experimental setting does not influence the mesenteric venous blood flow as previously demonstrated in animal models[30,31]. This phenomenon could be related to refractivity to volume replacement and vasopressor support[32,33]. The ongoing impairment of liver perfusion additionally reduced the oxygen supply essential for survival of hepatocytes and liver regeneration.

As the current model resulted in 100% mortality, it makes the identification of any regenerative process difficult to ascertain. This could mean that the decline in PBF observed during the ALF course could be misinterpreted as a start of systemic and hepatic restoration. However, firstly the hemodynamic situation of

recovering animals would certainly be stabilized and secondly the decrease of PBF, when liver restoration occurred, has already been described as a comparatively slow process in contrast to the rapid decline in the end stage of ALF[28].

It has recently been demonstrated experimentally[27,34] and clinically[35,36] that thrombocytes are able to promote liver regeneration after extended hepatectomy or liver transplantation. Their predictive value in the context of ALF remains unclear. Thrombocyte activation and increased fibrinolysis, highly suggestive of disseminated intravascular coagulation, occurs in ALF and may be related to endotoxemia or the release of thromboplastic material from the damaged liver.

The diagnosis remains difficult to substantiate as plasma concentrations of both fibrinogen and fibrin degradation products may be altered by ALF induced impairment of all serum coagulation parameters. Thrombocytopenia is a common finding in ALF from any cause[37]. Secondary to acetaminophen overdose it has been reported in a number of patients[38]. Clinical cases of acetaminophen intoxication with a plasma level of 250 mg/L ten hours after an intentional overdose of 50 g Paracetamol® tablets resulted in severe thrombocytopenia without accompanying anaemia or leucopenia approximately 48 h after ingestion[39]. A retrospective analysis of 174 patients by Fischereder and Jaffe[40] demonstrated that thrombocytopenia occurring early in the course of acetaminophen overdose was not uncommon and may identify a subset of patients with a high risk of hepatotoxicity. Our findings of a secondary sudden decline of thrombocytes following elevated acetaminophen plasma level (300-450 mg/L) support this hypothesis.

We conclude that the distinctive pattern of PBF during the intoxication process and subsequent ALF represents a reliable parameter for acetaminophen toxicity. Declining PBF in combination with ongoing worsening of hemodynamics and organ function precedes a fatal outcome after acetaminophen intoxication. An additional secondary sudden appearance of severe thrombocytopenia in ALF was found to reliably precede fatality within few hours.

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COMMENTS

Background

Several prognostic models have been invented to predict the outcome of patients suffering from acute liver failure (ALF). The crucial question is whether the liver regenerates spontaneously or if liver transplantation will be unavoidable. Large models of acetaminophen intoxication have been established in pigs recently.

Research frontiers

Based on observations of a preliminary animal study of acetaminophen intoxication in which a distinctive pattern of portal blood flow (PBF) and alterations in thrombocyte count were measure, the following animal study was aimed to characterize the clinical course of PBF and changes in thrombocyte count during the development, onset and further course of acetaminophen induced ALF.

Innovations and breakthroughs

This study verifies the expected distinctive pattern of PBF during the course of acetaminophen induced ALF: a considerable increase of PBF at manifestation of ALF followed by a rapid decline of PBF until end of the study. A parallel slow decline of thrombocyte values at manifestation of ALF was observed. Subsequent severe thrombocytopenia resulted in death due to multiple organ failure within few hours.

Applications

Declining portal blood flow during the course of ALF precedes fatality. Additional sudden appearance of severe thrombocytopenia leads to death due to multiple organ failure within few hours.

Peer-review

This paper highlights very important issue on acetaminophen intoxication. This is very current problem for the humans. The investigations were performed on a porcine model, not mice or other small vertebrates, which make them very valuable.

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ORIGINAL ARTICLE

Basic Study

Proton pump inhibitor induced collagen expression in colonocytes is associated with collagenous colitis

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Abstract

AIM

To elucidate the role of proton pump inhibitors (PPIs) in collagenous disease, direct effect of PPI on colonocytes was examined.

METHODS

Collagenous colitis is a common cause of non-bloody, watery diarrhea. Recently, there has been increasing focus on the use of proton PPIs as a risk factor for developing collagenous colitis. Mouse CT26 colonic cells were treated with PPI and/or PPI-induced alkaline media. Expression of fibrosis-associated genes was examined by RT-PCR. In human materials, collagen expression was examined by immunohistochemistry.

RESULTS

CT26 cells expressed a Na^+-H^+ exchanger gene (solute carrier family 9, member A2). Treatment with PPI and/or PPI-induced alkaline media caused growth inhibition and oxidative stress in CT26 cells. The treatment increased expression of fibrosis inducing factors, transforming growth factor β and fibroblast growth factor 2. The treatment also decreased expression of a negative regulator of collagen production, replication factor C1, resulting in increased expression of collagen types Ⅲ and Ⅳ in association with lipid peroxide. In biopsy specimens from patients with collagenous colitis, type Ⅲ and Ⅳ collagen were increased. Increase of type Ⅲ collagen was more pronounced in PPI-associated collagenous colitis than in non-PPI-

associated disease.

CONCLUSION
From these findings, the reaction of colonocytes to PPI might participate in pathogenesis of collagenous colitis.

Key words: Proton pump inhibitor; Collagenous colitis; pH; Fibrosis; Oxidative stress

Core tip: The main contribution of our paper is the finding of the basic mechanism of proton pump inhibitor evoking collagenous colitis with direct effects to colon epithelial cells. The collagenous colitis is a major cause of non-hemorrhagic watery diarrhea; however, the mechanism of the disease has not been fully elucidated. Our research findings show that proton pump inhibitor causes oxidative stress and collagen synthesis in colon epithelial cells, which might provide an impact to understanding pathogenesis of collagenous colitis and the side effect of proton pump inhibitor.

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INTRODUCTION

Collagenous colitis is one type of microscopic colitis and is the most common cause of non-bloody, watery diarrhea[1,2]. Originally Freeman *et al*[3] reported as a colitis with watery diarrhea and a lesion of colonic basement membrane. Collagenous colitis is characterized by collagenous bands at the basement membrane under microscopic examination[4,5]. This disease is treated with anti-inflammatory agents including salicylate[6]; however, untreated disease might progress into ulcerative colitis[7,8].

Recently, proton pump inhibitors (PPIs), especially lansoprazole, have been considered a potential cause of collagenous colitis[5]. Lansoprazole administration caused collagen bands in colonic mucosa in Mongolian gerbils[9]. However, the role of PPIs in the pathogenesis of collagenous colitis is not fully understood. Many pathologic mechanisms are proposed: colonic contents, mucosal immunity including autoimmunity and flora-associated immunity, eosinophilic reaction, human leukocyte antigen, biliary acids, anaerobic bacteria, fibroblast activation, and reduction of acidity in colonic contents due to suppression of acid secretion to the gastric juice[5,10]. In these candidates, the role of colonocytes' response to PPI is not given adequate attention. In the present study, we attempt to elucidate the pathologic importance of the direct effect of PPIs on colonocytes.

MATERIALS AND METHODS

Surgical specimens

We reviewed the pathology and clinical data of 11 patients with collagenous colitis diagnosed in the Department of Molecular Pathology, Nara Medical University from 2012 to 2015. Two samples of non-pathological colonic mucosa were used as controls. Basic patient information is summarized in Table 1. As written informed consent was not obtained, any identifying information was removed from the samples prior to analysis, in order to ensure strict privacy protection. All procedures were performed in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government, which was approved by the Ethics Committee of Nara Medical University (Approval Number 937).

Cell lines

CT26 mouse colon cancer cell line was provided by Professor I. J. Fidler (MD Anderson Cancer Center, Texas University). Cells were cultured in Dulbecco's modified Eagle's medium [DMEM; with high glucose (450 mg/dL), low glucose (100 mg/dL) or no glucose; WAKO Purechemical Ind. Ltd., Osaka, Japan), supplemented with 10% fetal bovine serum (FBS, Sigma Chemical Co., St. Louis, MO, United States) at 37 ℃ in 5% CO_2. Alkaline (pH 8.0) medium was made from DMEM with addition of Tris-HCl (pH 9.0, Sigma). Cell growth was assessed using a tetrazolium dye assay (MTT, Sigma), as previously described[11].

Reagents and enzyme-linked immunosorbent assay

Pantoprazole (Sigma), MitoGreen (Takara Bio Inc., Kusatsu, Japan), and 4-hydroxynonenal (4HNE) ELISA kit (R&D Systems Inc., Minneapolis, MN, United States) were purchased. For evaluation of MitoGreen, strength of fluorescence was measured in computer-captured images by using Photoshop Image Analyzer (Adobe Systems Inc., San Jose, CA, United States). 4HNE was measured in cell lysate according to the manufacturers' instructions. Whole-cell lysates were prepared using 0.1% nonidet 40 containing lysis buffer as previously described[12].

Immunohistochemistry

Consecutive 4-μm sections were immunohistochemically stained using the immunoperoxidase technique described previously[13]. Anti-type Ⅳ collagen antibody and anti-type Ⅲ collagen antibody (Abcam, Cambridge, MA, United States) were used at a concentration of 0.2 μg/mL. Secondary antibodies (Medical and

Table 1 List of primer sets for RT-PCR

Gene	Symbol	Accession No.	Upper primer	Lower primer
Collagen type Ⅳ	*Col4a1*	NM_009931.2	AAAGGGAGAAAGAGGCTTGC	CCTTTGTACCGTTGCATCCT
Collagen type Ⅵ	*Col6a1*	NM_009933.4	GATGAGGGTGAAGTGGGAGA	CAGCACGAAGAGGATGTCAA
Collagen type Ⅰ	*Col1a1*	NM_007742.4	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
Collagen type Ⅲ	*Col3a1*	NM_009930.2	GCACAGCAGTCCAACGTAGA	TCTCCAAATGGGATCTCTGG
Solute carrier family 9, member A2	*Slc9a2*	NM_001033289.2	ACTGGGGTCACAACTTCTGG	CTTCACGGCAGTCATTGAGA
Transforming growth factor β1	*Tgfb1*	NM_011577.2	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC
Fibroblast growth factor 2	*Fgf2*	NM_008006.2	AGCGGCTCTACTGCAAGAAC	GCCGTCCATCTTCCTTCATA
Replication factor C1	*Rfc1*	NM_011258.2	TGATCACAGGAGTGCTGGAG	CGAGGATTTTTGTTCCCAGA
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	NM_001289726.1	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA

Biological Laboratories, Nagoya, Japan) were used at a concentration of 0.2 μg/mL. Tissue sections were color-developed with diamine benzidine hydrochloride (DAKO, Glastrup, Denmark), and counterstained with Meyer's hematoxylin (Sigma). We evaluated immunopositivity at the basement membrane and stromal fibers. Staining strength was scored from (-) to (++): (-) is no staining; (+) is staining equal to that of non-pathologic mucosa; (++) is staining more pronounced than that of non-pathologic mucosa. All samples were stained at one time to equalize staining conditions. For a negative control, unimmunized rat IgG (Santa-Cruz Biotechnology, Santa-Cruz, CA, United States) was used as primary antibody.

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) of 0.5 μg total RNA extracted using an RNeasy kit (Qiagen, Germantown, MD, United States) was performed. Primer sets used in the experiment are listed in Table 1. Primers were synthesized by Sigma Genosys (Ishikari, Japan). PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide.

Statistical analysis

Statistical significance was calculated by using two-tailed Fisher's exact, Chi-square, and unpaired Student-*t* tests by using InStat software (GraphPad, Los Angeles, CA, United States). Statistical significance was defined as a two-sided *P*-value of < 0.05.

RESULTS

Effect of PPI and/or alkaline pH on cell growth of CT26 cells

The effect of PPIs was examined by using CT26 mouse colon cancer cells treated with pantoprazole and/or alkaline media[14] (Figure 1). PPI treatment did not affect cell proliferation of CT26 cells regardless of glucose concentration (Figure 1A). In contrast, alkaline media suppressed cell proliferation at 100 and 450 mg/dL glucose concentrations (Figure 1B). When treated with PPI and alkaline media together, cell proliferation in 0 and 450 mg/dL glucose concentrations was suppressed more than in 100 mg/dL glucose concentration (Figure 1C).

Effect of PPI and/or alkaline pH on oxidative stress and mitochondria volume

Next, oxidative stress was examined by 4HNE levels, as it is a marker for lipid peroxide (Figure 2A and B). Treatment with PPI or alkaline media increased levels of 4HNE in various glucose concentrations. Alkaline media-treated cells showed higher 4HNE levels than PPI-treated cells. Treatment with PPI or alkaline media decreased mitochondrial volume (Figure 2C), particularly in 100 and 450 mg/dL glucose concentrations compared to 0 mg/dL glucose concentration (Figure 2D).

Expression of collagen and collagen-associated genes in CT26 cells

We confirmed the expression of Na^+-H^+ exchanger (solute carrier family 9, member A2; *SLC9A2*), a pharmacological target of PPIs (Figure 3A). *SLC9A2* expression was not affected by treatment with PPI or alkaline media. Expression of transforming growth factor (TGF) β and fibroblast growth factor (FGF) 2 was increased by treatment with PPI or alkaline media. TGF-β and FGF2 are known to stimulate collagen-producing fibroblasts[14]. In contrast, expression of replication factor C1 (*RFC1 orAlp145*), a negative regulator of collagen expression[15], was downregulated by treatment with PPI or alkaline media. Compared to other collagen types, mRNA expression of type Ⅲ and Ⅳ collagen was increased in CT26 cells treatment with PPI or alkaline media (Figure 3B-D).

Production of collagen type Ⅲ and Ⅳ in human collagenous colitis mucosa

We confirmed the increase of collagen type Ⅲ and Ⅳ in human colonic mucosa of patients with collagenous colitis (Figure 4). HE and Azan staining showed thickening of the basement membrane at the covering epithelium (Figure 4A-C). Immunohistochemistry of collagen type Ⅲ showed that collagen fibers were increased at the basement membrane and mucosal stroma when compared with non-pathologic mucosa (Figure 4D and E). In contrast, immunohistochemistry of collagen type Ⅳ showed that collagen fibers were increased at the basement membrane alone when

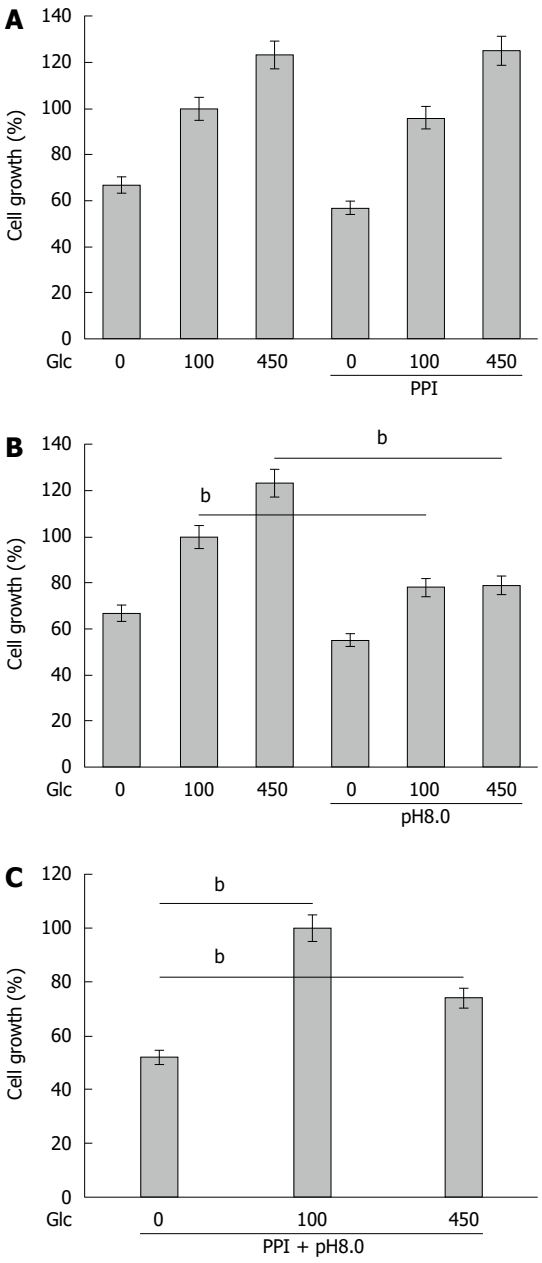

Figure 1 Effect of proton pump inhibitor and/or alkaline pH on CT26 cell growth. Cell growth of CT26 cells was examined in different concentration of glucose (Glc) concentration (0, 100, 450 mg/dL). A: Pantoprazole (10 μg/mL) treatment (PPI); B: DMEM-pH8.0 treatment (pH 8.0); and C: Treatment with PPI and alkaline media (pH 8.0). Error bar, SD calculated from 3 independent experiments by Student's *t*-test. PPI: Proton pump inhibitor. $^{b}P < 0.001$.

compared with non-pathologic mucosa.

Finally, expression of collagen type Ⅲ and Ⅳ was compared in 11 collagenous colitis patients (Tables 2 and 3). The levels of collagen type Ⅲ were increased in PPI-associated collagenous colitis whereas levels of collagen type Ⅳ were not different between PPI-associated and non-PPI-associated collagenous colitis.

DISCUSSION

Collagenous colitis is an increasingly common microscopic colitis[1]. Recently proton pump inhibitors, especially lansoprazole, are recognized to be associated with onset of collagenous colitis[5]. Thus, the mechanism of this association is a focus of studies on collagenous colitis. In the present study, we examined the direct effect of PPIs on colonocytes, as well as the effect of increased pH of colonic contents.

We confirmed that the expression of the SLC9A2 Na^{+}-H^{+} exchanger is a target of PPIs in the colonocytes. PPIs did not affect CT26 cell growth but alkaline conditions did suppress cell growth. Growth suppression of colonocytes might induce retardation of wound healing causing subsequent persistent inflammation, immune disturbances, and collagen overproduction.

PPI and alkaline condition caused increase of lipid peroxide with decrease of mitochondrial volume. Inhibition of proton efflux might induce acidic alteration of the cytoplasm, which might affect oxidative phosphorylation in mitochondria. Monitoring of mitochondrial function, ATP production and mitochondrial voltage are needed to conclude the effect of PPI on oxidative stress.

PPI and alkaline condition decreased the expression of RFC1, which is a negative regulator of collagen gene expression[16]. We then examined the alteration of expression of collagen type Ⅰ, Ⅲ, Ⅳ and Ⅵ. Only collagen type Ⅲ and Ⅳ showed increase of mRNA levels in cells treated with PPI or alkaline conditions. In colon biopsy specimens of collagenous colitis patients, the amounts of type Ⅲ and type Ⅳ collagens were

Table 2 Expression of collagen Ⅲ and Ⅳ in collagenous colitis

Case no.	Age	Sex	PPI	Expression	
				Collagen Ⅲ	Collagen Ⅳ
1	79	F	+	++	++
2	71	F	+	+++	++
3	87	F	+	++	++
4	26	F	+	+++	++
5	80	M	+	+++	+
6	78	M	+	+++	++
7	80	F	-	++	+
8	45	M	-	+	++
9	67	M	-	++	+
10	59	F	-	+	++
11	77	F	-	++	++

PPI: Proton pump inhibitor.

Table 3 Expression of collagen type Ⅲ and Ⅳ in proton pump inhibitor-asscoiated collagenous colitis

	PPI (+)	PPI (-)	*P* value
n	6	5	
Age	70.2	65.6	NS
M:F	2:4	2:3	NS
Collagen			
Type Ⅲ	2.67 ± 0.52	1.6 ± 0.55	0.0089
Type Ⅳ	1.83 ± 0.41	1.6 ± 0.55	NS

PPI: Proton pump inhibitor.

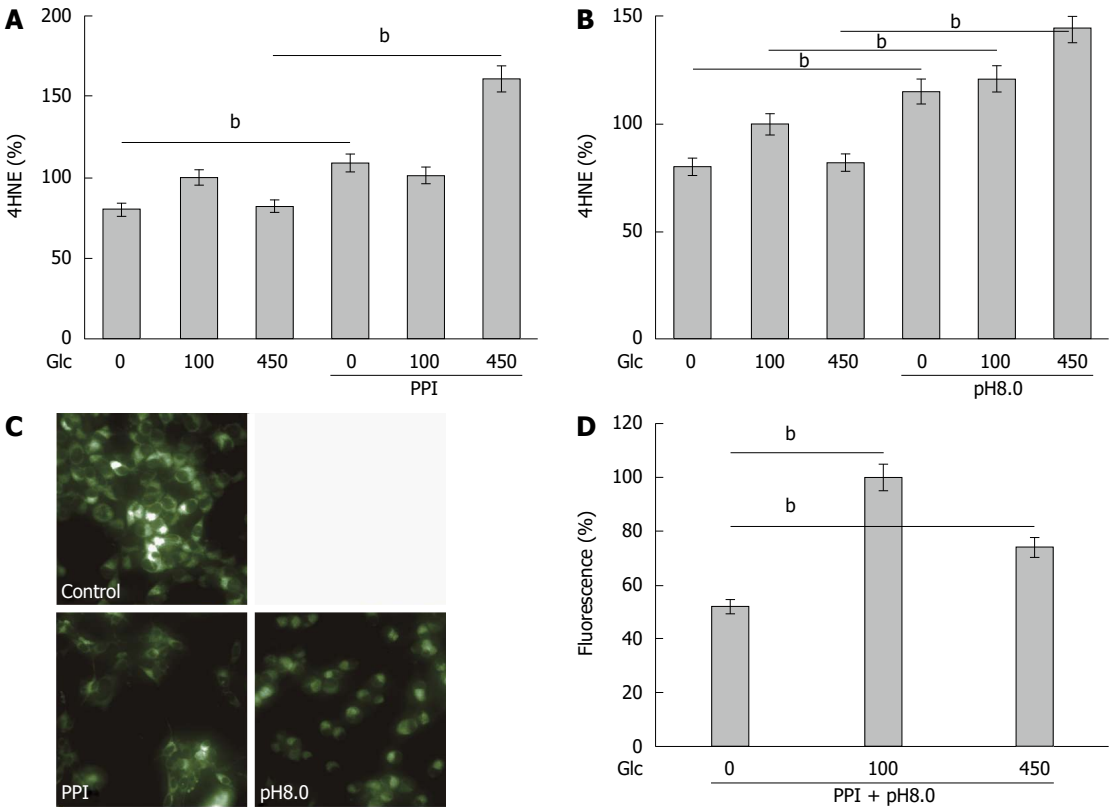

Figure 2 Effect of proton pump inhibitor and/or alkaline pH on oxidative stress and mitochondria volume. A and B: Levels of 4-hydroxynonenal (4HNE) was measured by ELISA. Relative quantities were designated (Glucose 100 mg/dL without PPI or pH8.0 is set to 100%); C: Mitochondrial volume was examined by Mitogreen staining; D: The fluorescence strength of Mitogreen (Strength in cells treated with glucose 0 mg/dL is set to 100%). Error bar, SD calculated from 3 independent experiments by Student's *t*-test. PPI: Proton pump inhibitor. $^{b}P < 0.001$.

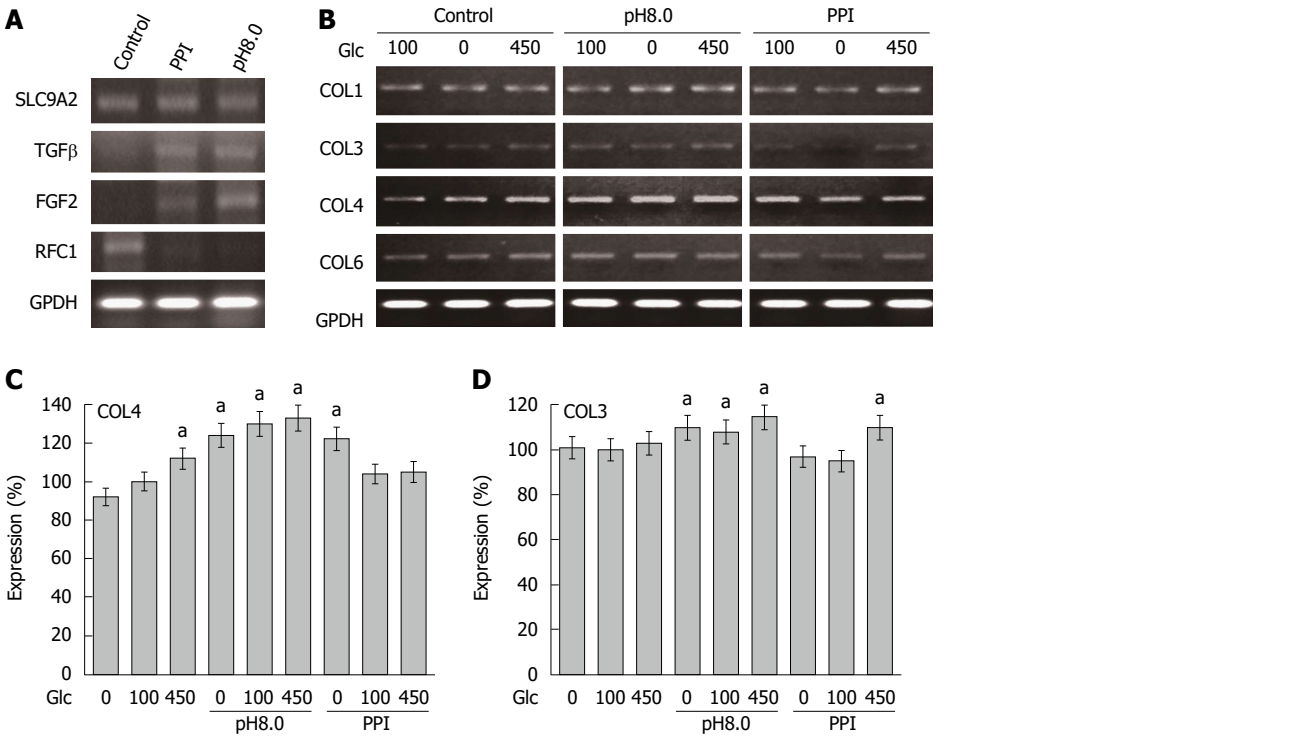

Figure 3 Expression of collagen and collagen-associated genes in CT26 cells. A: Expression of Na^{+}-H^{+} exchanger gene (SLC9A2) and collagen-associated genes was examined by RT-PCR in CT26 cells treated with PPI or alkaline media. Glyceraldehyde 3-phosphate dehydrogenase (GPDH) served as an internal control; B: Expression of collagen type Ⅰ, Ⅲ, Ⅳ and Ⅵ in CT26 cells treated with PPI or alkaline media; C and D: The expression of collagen type Ⅰ, Ⅲ, Ⅳ and Ⅵ in CT26 cells was semi-quantitated. Expression in cells without treatment in 100 mg/dL glucose concentration was set to 100% as a reference. Asterisk shows significant difference from the reference. Error bar, SD calculated from 3 independent experiments by Student's *t*-test. PPI: Proton pump inhibitor.

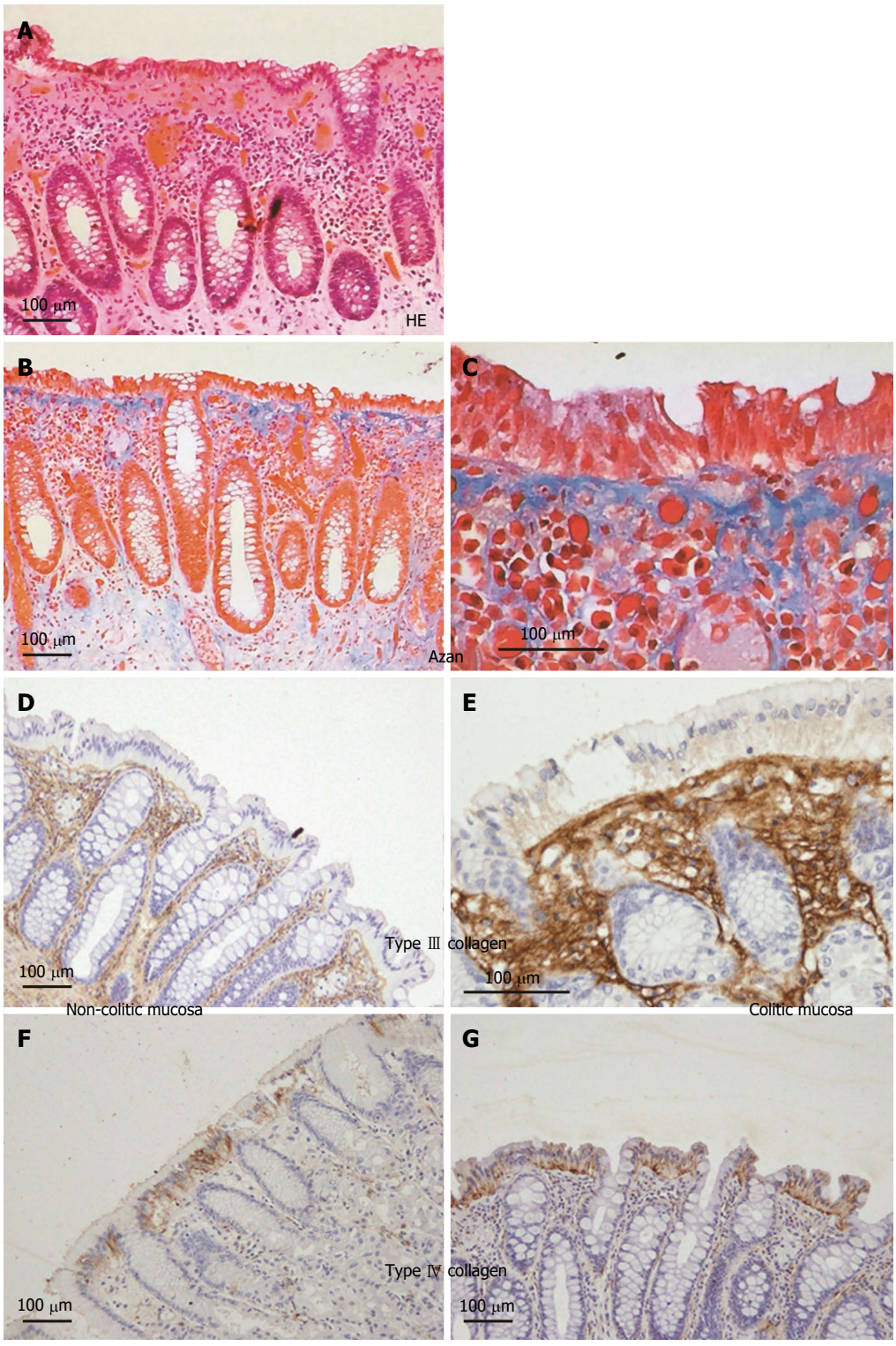

Figure 4 Expression of collagen type Ⅲ and Ⅳ in colonic mucosa of collagenous colitis patients. A-C: Histopathological appearance of colonic mucosa in collagenous colitis patients. Thickened basement membrane at the covering epithelium was observed in HE (A) and Azan (B and C) staining; D-G: Immunohistochemistry of collagen type Ⅲ (D and E) and type Ⅳ (F and G) in non-pathologic mucosa (D and F) and collagenous colitis mucosa (E and G).

increased. Type Ⅲ collagen was increased in the basement membrane and mucosal stroma, whereas type Ⅳ collagen was increased only in the basement membrane. Type Ⅳ collagen is a major component of basement membrane collagens[17]. The expression level of type Ⅲ collagen in PPI-associated patients was higher than in non-PPI-associated patients. These discrepancies between our data and Aigner's report might be due to differences in the pathogenesis of collagen disease or due to the influence of PPIs.

We examined pantoprazole in this study. To determine the significance of lanzoprazole in occurrence of

collagenous colitis, we examine several kinds of PPIs in future. NSAIDs are also associated with collagenous colitis in cases. The mechanism is also examined to compare with that of PPIs.

In our data, PPI and alkaline condition increased the expression of TGF-β and FGF2, which are well known fibrosis-inducing factors[15]. We focused on the direct effect of PPI in colonocytes; however, stromal cells, such as fibroblasts are also involved in collagen production in collagenous colitis[5]. Our results suggest that PPIs induce fibroblast-mediated collagen production *via* stimulation of colonocyte secreting TGF-β and FGF2.

In the literature, alteration of gut flora is relevant for development of collagen colitis; clostridium, in particular, is associated with the disease[18-20]. Reduction of acidity in colonic contents by PPIs activates clostridium virulence[10]. The flora alteration might induce collagen production in the colonic mucosa. Anaerobic bacteria, including clostridium, induce TGF-β expression in colonocytes, which enhances collagen deposition in the colonic wall[21]. The TGF-β induction is caused by activation of the mucosal immunity[22]. Interleukin-13 increase in the inflammatory mucosa is reported to activate TGF-β expression[23]. Alkaline conditions might also affect gut flora, whose imbalance is regarded as important in causing collagenous colitis.

These findings suggest that collagen production and expression of collagen producing factors are directly stimulated by PPIs and PPI-induced alkaline change of colonic content. Colonocyte response to these factors might contribute to the pathogenesis of collagenous colitis.

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COMMENTS

Background

Collagenous colitis is a clinical problem recognized recently in an association with administration with proton pomp inhibitor (PPI). Since the pathogenesis is still controversy, reveal of the disease mechanism might be expected to develop a new therapy.

Research frontiers

Many recent studies show the immunological and bacteriological effect of PPI. Study on the direct effect of PPI on colonocytes is a novel approach.

Innovations and breakthroughs

PPI and the PPI-resulted alkaline condition altered mitochondrial energy metabolism and collagen expression in colonocytes.

Applications

PPI-induced oxidative stress is not only a possible cause of collagen production in colon epithelium but also an important factor affecting tumor growth or function of mitochondria-rich tissues, such as myocardium, hepatocytes.

Peer-review

Interesting paper on possible mechanism for lanzoprazole associated collagenous colitis. It would be very interesting to evaluate other PPIs, such as omeprazole (a much rarer association with collagenous colitis), as well as with other agents (*e.g.*, non-steroidal antiinflammatory drugs) to determine if this mechanism is shared with other agents.

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ORIGINAL ARTICLE

Basic Study

miR-1181 inhibits invasion and proliferation *via* STAT3 in pancreatic cancer

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Author contributions: Wang J and Guo XJ contributed equally to this work; Wang J performed the majority of experiments and analyzed the data; Guo XJ performed the molecular investigations; Guo XJ, Ding YM and Jiang JX designed and coordinated the research; Wang J, Guo XJ and Jiang JX wrote the paper.

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Abstract

AIM

To examine the role of microRNA 1181 (miR-1181) in invasion and proliferation in pancreatic cancer.

METHODS

We analyzed the expression of miR-1181 in several pancreatic cancer cell lines and generated stable MIA-PaCa-2 and PANC-1 cell lines with up-regulated miR-1181 expression using an adenovirus delivery system. We then investigated miR-1181's effect on invasion and proliferation of pancreatic cancer cells by transwell assay, wound healing assay, cell counting kit-8 assay and colony-forming assay, and explored any underlying mechanisms by western bolt. Beyond that, we observed the change of the PANC-1 cell's cytoskeleton by immunofluorescence staining.

RESULTS

Our data showed that miR-1181 was relatively down-regulated in pancreatic cancer cell lines compared with normal pancreatic ductal epithelial cells. And miR-1181 inhibited the migration, invasion and proliferation activities of MIA-PaCa-2 and PANC-1 cells. Notably,

after over-expressing of miR-1181 in PANC-1 cells, F-actin depolymerized. Immunofluorescence staining shows decreased F-actin and β-tubulin expression in PANC-1 cells over-expressing miR-1181 compared with the control cells. Furthermore, we found that over-expressing miR-1181 inhibited the expression of signal transducer and activator of transcription 3 (STAT3) while knocking-down miR-1181 up-regulated the expression of STAT3. Knocking-down miR-1181 promoted the invasion and proliferation of pancreatic cancer cells. And inhibition of STAT3 blocked the promotion effects of knocking-down miR-1181 on proliferation and invasion in pancreatic cancer.

CONCLUSION

Together our findings suggest that miR-1181 may be involved in pancreatic cancer cell invasion and proliferation by targeting STAT3 and indicate that miR-1181 may be a potential therapeutic agent for pancreatic cancer.

Key words: Pancreatic cancer; miR-1181; Proliferation; Invasion; STAT3

Core tip: We found that miR-1181 may be involved in pancreatic cancer cell invasion and proliferation by targeting signal transducer and activator of transcription 3. Our findings suggest that miR-1181 may be a potential therapeutic agent for pancreatic cancer.

Wang J, Guo XJ, Ding YM, Jiang JX. miR-1181 inhibits invasion and proliferation *via* STAT3 in pancreatic cancer. *World J Gastroenterol* 2017; 23(9): 1594-1601 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1594.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1594

INTRODUCTION

Pancreatic cancer is a highly lethal disease, with a mortality that closely parallels incidence. Most patients with pancreatic cancer remain asymptomatic until the disease reaches an advanced stage. Surgical resection is currently regarded as the only potentially curative treatment[1-3]. Therefore, there is an urgent need to design novel strategies for achieving better treatment outcome in pancreatic cancer patients. High invasion and rapid proliferation are the two main features of pancreatic cancer tumors[4]. Understanding the molecular mechanisms that collaboratively regulate pancreatic cancer cell invasion and proliferation is expected to provide new insights into the development of novel and effective therapies for pancreatic cancer.

MicroRNAs (miRNAs) are noncoding 17- to 25-nucleotide-long RNAs that posttranscriptionally regulate the expression of multiple genes[5]. miRNA dysregulation is implicated in the development and progression of nearly all tumor types[6-8]. Recent evidence has indicated that some miRNAs can function as tumor suppressors[9]. We previously found that miR1181 inhibited CSC phenotypes by directly suppressing SOX2 and STAT3 in pancreatic cancer cells[10]. But we have not elucidated miR-1181's role on proliferation and invasion of pancreatic cancer cells, which was important malignant biological behavior of pancreatic cancer. And CSCs regulator always play an important role in the process of growth and invasion. As a result, we hypothesized that miR-1181 may suppress the invasion and proliferation of pancreatic cancer and explored it.

Table 1 Primers used in real-time polymerase chain reaction

Gene name		Sequences (forward and reverse)
GAPDH	F	TGACTTCAACAGCGACACCCA
	R	CACCCTGTTGCTGTAGCCAAA
STAT3	F	AGTGACCAGGCAGAAGATGC
	R	CTCTTCCAGTCAGCCAGCTC

MATERIALS AND METHODS

Cell lines and cultures

The HPDE, BxPC-3, AsPC-1, MIA-PaCa-2 and PANC-1 cell lines were purchased and authenticated though STR typing from ATCC. MIA-PaCa-2 and PANC-1 cells were grown in DMEM medium (Gibco, NY, United States) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin G and 100 μg/ml streptomycin (Sigma, MO, United States) at 37 ℃ in a humidified 5% CO_2 incubator. HPDE, BxPC-3 and AsPC-1 cells were grown in 1640 medium (Gibco) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G and 100 μg/mL streptomycin at 37 ℃ in a humidified 5% CO_2 incubator.

RNA preparation and real-time PCR

Harvesting, tissue processing, and reactions were conducted according to standard procedures. RNA was reverse-transcribed to cDNA using the ReverTra Ace qPCR RT Kit (TransGen, China). Quantitative real-time PCR was performed using the SYBR Green Realtime PCR Master Mix (TransGen), according to the manufacturer's instructions; and primers were shown in Table 1.

Generation of stable cell lines

We purchased human miR-1181 over-expressing (miR-1181U), knocking-down (miR-1181D) and negative-control (NC) adenovirus from Genechem (Shanghai, China). All transfections were carried out according to manufacturers' instructions.

In vitro invasion and migration assays

Transwell assay: Cell invasion and migration were

assessed using 24-well Corning Costar inserts with 8-μm pores. The upper surface of each insert was coated with Matrigel (BD, NJ, United States; diluted 1:8) for 6 h in an incubator. Cells (1×10^5) were added to upper chambers and incubated at 37 ℃; migration was assessed at 12 h and invasion at 24 h. Non-migrating and non-invading cells were removed with cotton buds from the top chambers. Cells remaining in bottom chambers were fixed with 100% methanol, stained with 0.1% crystal violet in 2% ethanol, and quantified visually in nine random fields using bright-field optics. Experiments were performed in triplicate and data are reported as mean ± SD of cell numbers.

Wound healing assay: Cells (5×10^6 per well) were cultured in 6-well plates for 24 h. Cell layers were subsequently scratched with sterile plastic tips, washed with PBS twice, cultured for 24 h with medium containing 1% FBS, and photographed under an Olympus BX51 microscope.

Immunofluorescence

Cells grown on coverslips were incubated overnight at 4 ℃ with primary antibody against β-tubulin (CST; 1:100), followed by incubation for 45 min at 37 ℃ with CY3-conjugated goat anti-rabbit antibody (Boster, Wuhan, China). F-Actin distribution was detected using rhodamine phalloidin (Sigma-Aldrich) according to the manufacturer's protocol. Slides were counterstained with DAPI to visualize the cell nuclei, photographed using a LEICA LCSSP2 confocal laser scanning microscope and analyzed by ZEN 2009 (Carl Zeiss, Baden-Wurttemberg, Germany).

CCK-8 assay

Cells were seeded in 96-well culture plates (2×10^4 cells/100 μL/well) and grown in the incubator. We then added 10 μL of CCK-8 (Dojindo, Tokyo, Japan) solution to each well. Plates were incubated for 1 h in the incubator and the absorbance was monitored at 450 nm using a microplate reader.

Colony-forming assays

Cells were isolated by Trypsin-EDTA and seeded into 6-well plates at 1×10^3 cells per well in a final volume of 2 mL culture media. Cells were allowed to grow at 37 ℃ for 1 wk. After three washes with PBS, cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 30 min. Total colonies were counted from seven random fields under the microscope. Assays were repeated at least three times.

siRNA transfection

We purchased siRNAs from RiboBio (Guangzhou, China). Cells were seeded in 6-well plates at 50% confluence without antibiotics on the day before transfection. Transfection with si-STAT3 or miRNA negative control #22 (NC) (Ribobio, Guangzhou, China) was performed using Lipofectamine 2000 reagent (Invitrogen, NY, United States). Transfection complexes were prepared according to the manufacturer's instructions. All transfections were carried out according to manufacturers' instructions.

Western blot analysis

Polyvinylidene difluoride membranes containing electrophoretically separated proteins from PANC-1 and MIA-PaCa-2 cells were incubated with rabbit anti-STAT3 (CST, United States; 1:200), rabbit anti-p-STAT3 (CST, United States; 1:200), and mouse anti-GAPDH (Boster), followed by incubation with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (CST; 1:2000) and visualized by enhanced chemiluminescence (Boster).

Statistical analysis

All values are presented as means ± SD. Significant differences were determined using SPSS 17.0 software (SPSS, Chicago, IL, United States). Student's *t*-test was used to determine statistical differences. $P < 0.05$ was considered significant.

RESULTS

miR-1181 expression in pancreatic cancer cell lines

We first analyzed the expression of miR-1181 in several pancreatic cancer cell lines, including MIA-PaCa-2, BxPC-3, AsPC-1 and PANC-1, by qRT-PCR (Figure 1A). We found that the expression level of miR-1181 is downregulated in pancreatic cancer cell lines compared with levels in the human pancreatic duct epithelial (HPDE) cell line. We therefore generated stable MIA-PaCa-2 and PANC-1 cell lines with up-regulated miR-1181 expression using an adenovirus delivery system. Up-regulated miR-1181 (miR-1181U) was verified by PCR analyses (Figure 1B and C).

miR-1181 inhibits pancreatic cancer cell migration and invasion

To examine whether up-regulated miR-1181 affect MIA-PaCa-2 and PANC-1 cell migration, we performed transwell assays to examine whether miR-1181 affect MIA PaCa-2 and PANC-1 cell migration and invasion. We found that over-expression of miR-1181 inhibited MIA-PaCa-2 and PANC-1 cell migration and invasion (Figure 2A-F). We next performed wound healing assays. Our results showed that overexpression of miR-1181 inhibited both MIA-PaCa-2 and PANC-1 cell migration (Figure 2G-J). As expected, knockdown of miR-1181 resulted in suppressed invasiveness of MIA-PaCa-2 and PANC-1 cells. Together these data indicate that miR-1181 inhibits migration and invasion of MIA-PaCa-2 and PANC-1 cells *in vitro*. Notably, after over-expressing of miR-1181 in PANC-1 cells, F-actin depolymerized. Immunofluorescence staining shows

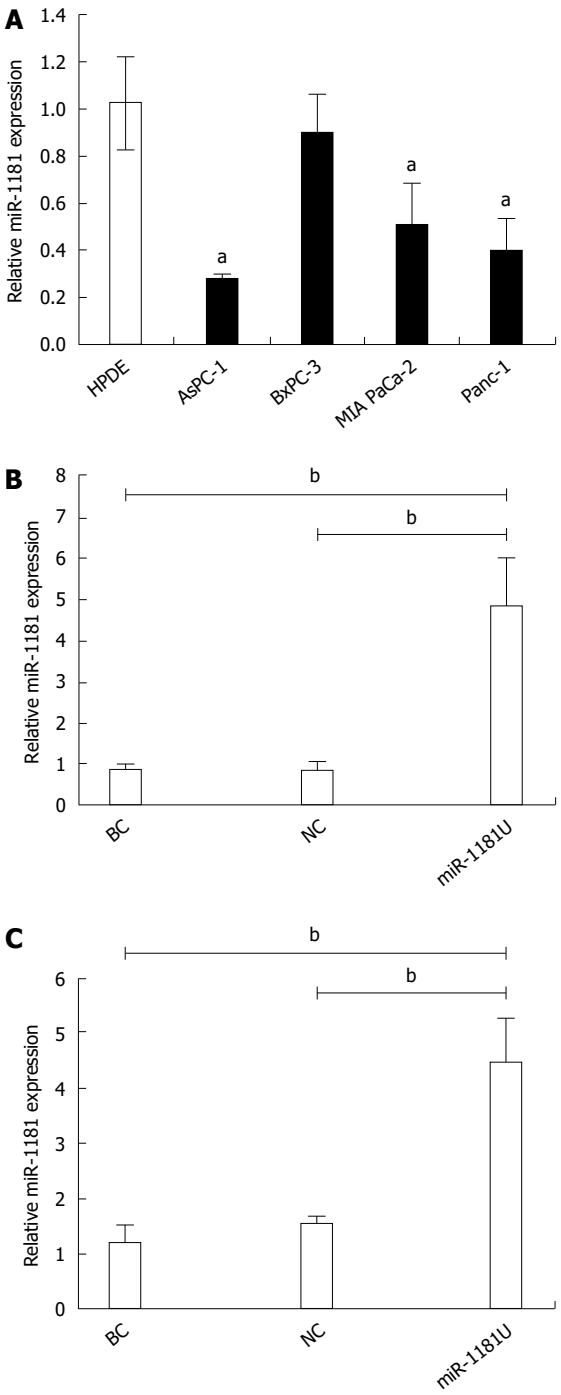


Figure 1 miR-1181 was downregulated in pancreatic cancer cell lines. A: MiR-1181 expression was evaluated in pancreatic cancer cell lines and HPDE cells by PCR, $^{a}P < 0.05$; B: Confirmation of miR-1181 upregulation in PANC-1 stable cell lines compared with negative control (NC) cells by PCR; C: Confirmation of miR-1181 upregulation in MIA-PaCa-2 stable cell lines compared with NC cells by PCR, $^{b}P < 0.01$. BC: Blank control.

decreased F-actin and β-tubulin expression in PANC-1 cells over-expressing miR-1181 compared with the control cells (Figure 2K).

miR-1181 inhibits pancreatic cancer cell proliferation

To examine whether miR-1181 expression affected MIA-PaCa-2 and PANC-1 cells proliferation, we next performed CCK-8 assays and colony forming assays. Our results showed that over-expression of miR-1181 inhibited MIA-PaCa-2 and PANC-1 cell proliferation in both CCK-8 assays (Figure 3A and B) and colony-forming assays (Figure 3C-F).

miR-1181 inhibits invasion and proliferation via STAT3 in human pancreatic cancer cells

We previously found that miR-1181 directly targets STAT3 and inhibits STAT3 transactivity. It was found that STAT3 activation may promote the progression of pancreatic cancer. Thus, we next examined whether miR-1181 promoted pancreatic cell invasion and proliferation though the STAT3 pathway. Firstly, we found that over-expressing miR-1181 inhibited the expression of STAT3 and p-STAT3 while knocking-down miR-1181 up-regulated the expression of STAT3 and p-STAT3 in western blot assay (Figure 4A). Next we generated stable MIA-PaCa-2 and PANC-1 cell lines with knocked-down miR-1181 expression using an adenovirus delivery system. We transfected knocked-down miR-1181 MIA-PaCa-2 and PANC-1 cells with siRNA targeting STAT3 and examined cell proliferation and invasion (Figure 4C-J). Our results showed that inhibition of STAT3 by siRNA blocked the promotion effects of knocking-down miR-1181 on proliferation and invasion.

DISCUSSION

This study demonstrated that miR-1181 expression is markedly downregulated in pancreatic cancer cells and that upregulation of miR-1181 could suppress the invasion and proliferation of pancreatic cancer cells. Moreover, our results suggest that miR-1181 inhibits invasion and proliferation through targeting STAT3 in pancreatic cancer cells. In conclusion, our results indicate that miR-1181 plays an important role in suppressing invasion and proliferation in pancreatic cancer though targeting STAT3.

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway was originally discovered in the context of interferon-α (IFNα)-, IFNγ- and intereukin-6 (IL-6)-mediated downstream signaling pathways[11]. Among the seven members of the STAT protein family, STAT3 and STAT5 are the most important for cancer progression[12,13]. Although both STAT3 and STAT5 contribute to tumor cell proliferation and survival, a notable feature of STAT3 as a promising target for cancer therapy is that it also has a crucial role in stromal cells, including immune cells, which are recruited to the tumor microenvironment to promote tumor progression. Importantly, STAT3 activation also functions as a potent immune checkpoint for multiple anti-tumor immune responses. Evidence continues to accumulate that STAT3 activation is a promising molecular target for cancer therapies[14-17]. The STAT3 signaling pathway is especially important

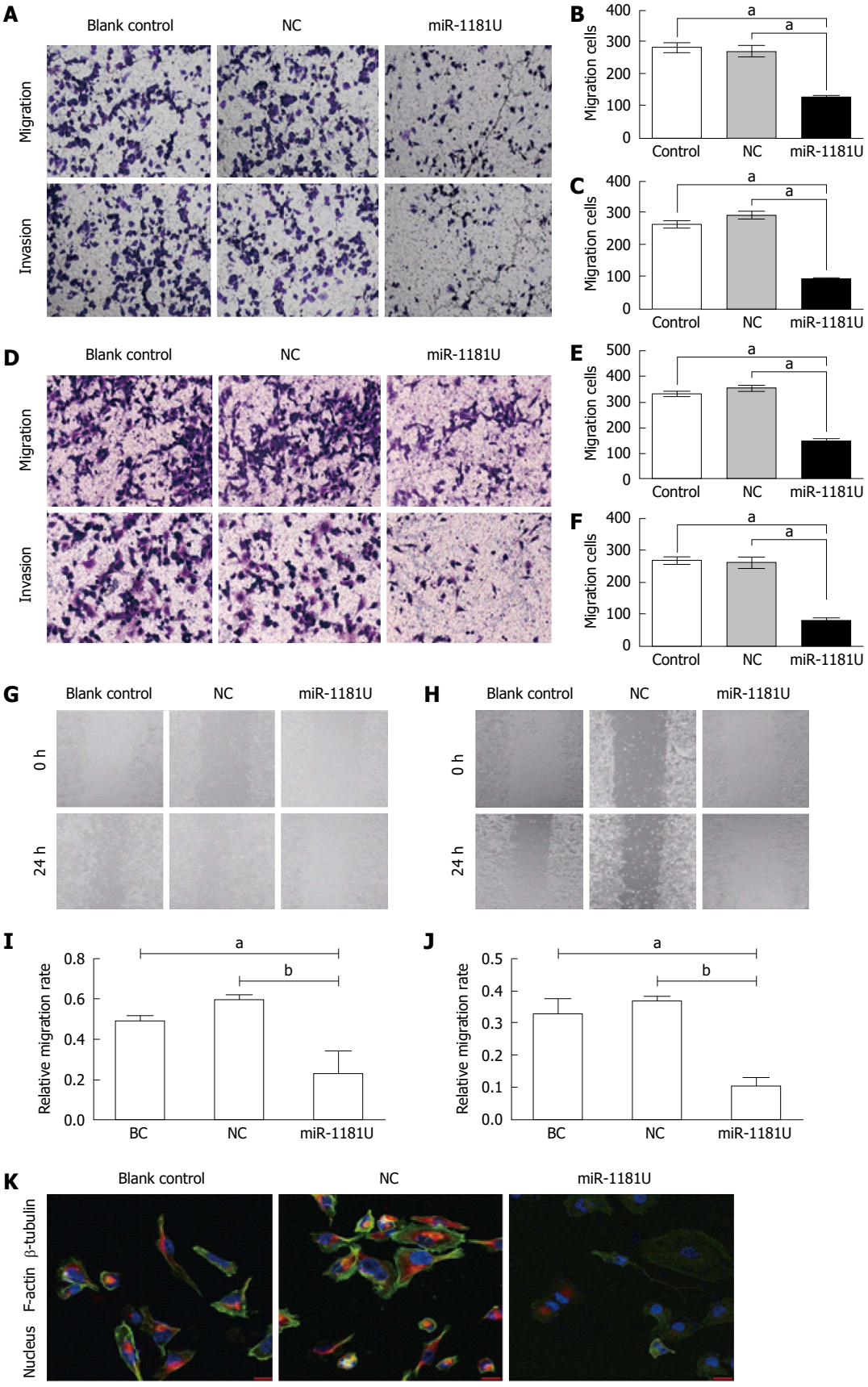


Figure 2 miR-1181 inhibited pancreatic cancer cell migration and invasion. A: Indicated PANC-1 cells were subjected to transwell assays and relative migration and invasion capability was quantitative analyzed (B and C), $^{a}P < 0.05$; D: Indicated MIA-PaCa-2 cells were subjected to transwell assays and relative migration and invasion capability was quantitative analyzed (E and F), $^{a}P < 0.05$; G and I: Indicated PANC-1 and MIA-PaCa-2 (H and J) cells were subjected to wound-healing assays and quantitative analyzed, $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$; K: Immunofluorescence staining shows decreased F-actin and β-tubulin expression in PANC-1 cells over-expressing miR-1181 compared with the blank control and negative control (NC) cells.

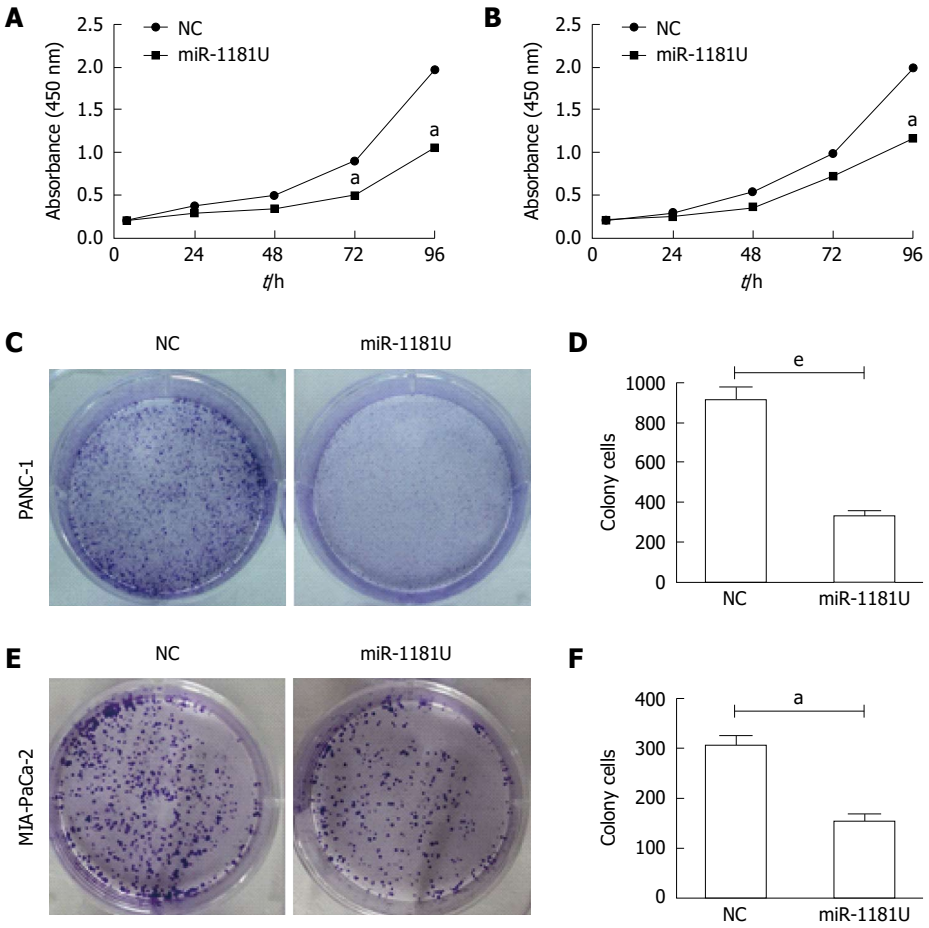
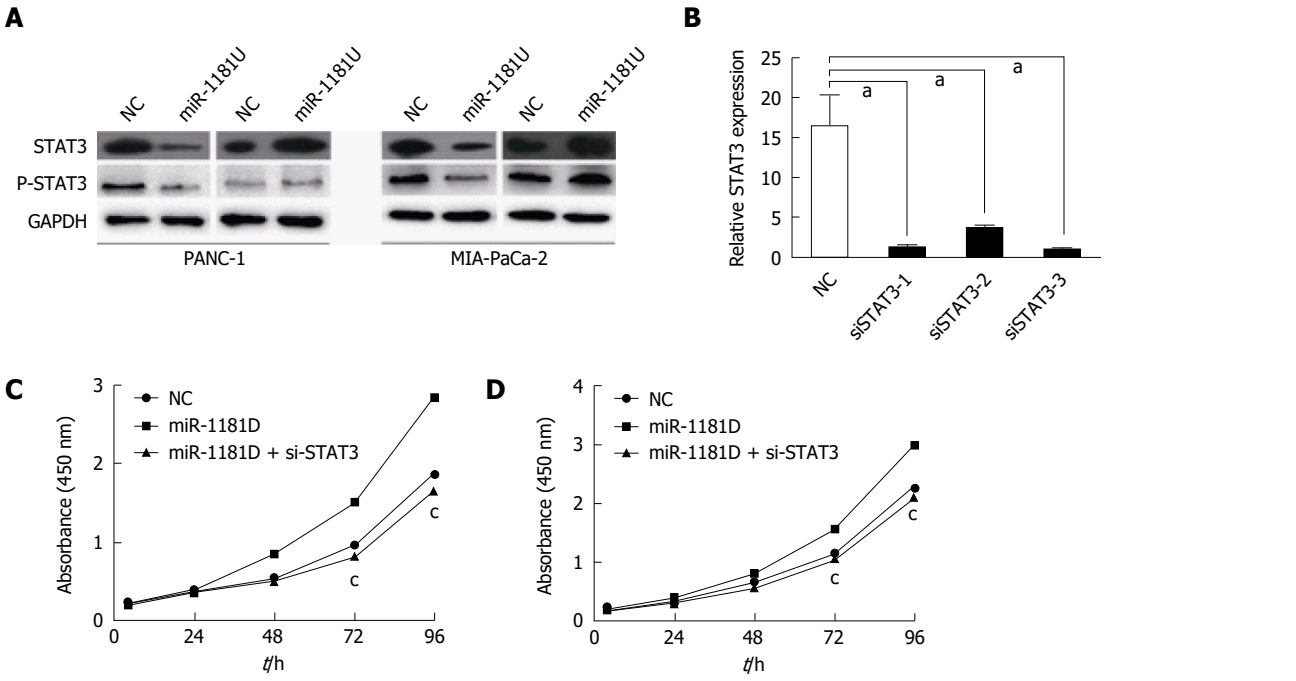


Figure 3 MiR-1181 inhibited pancreatic cancer cell proliferation. Indicated PANC-1 (A) and MIA-PaCa-2 (B) cells were subjected to CCK-8 assays, $^{a}P < 0.05$. Negative control (NC) cells were used as control. $^{a}P < 0.05$; C: Indicated PANC-1 cells were subjected to colony forming assays and quantitative analyzed (D), $^{e}P < 0.001$; E: Indicated MIA-PaCa-2 cells were subjected to colony forming assays and quantitative analyzed (F), $^{a}P < 0.05$.



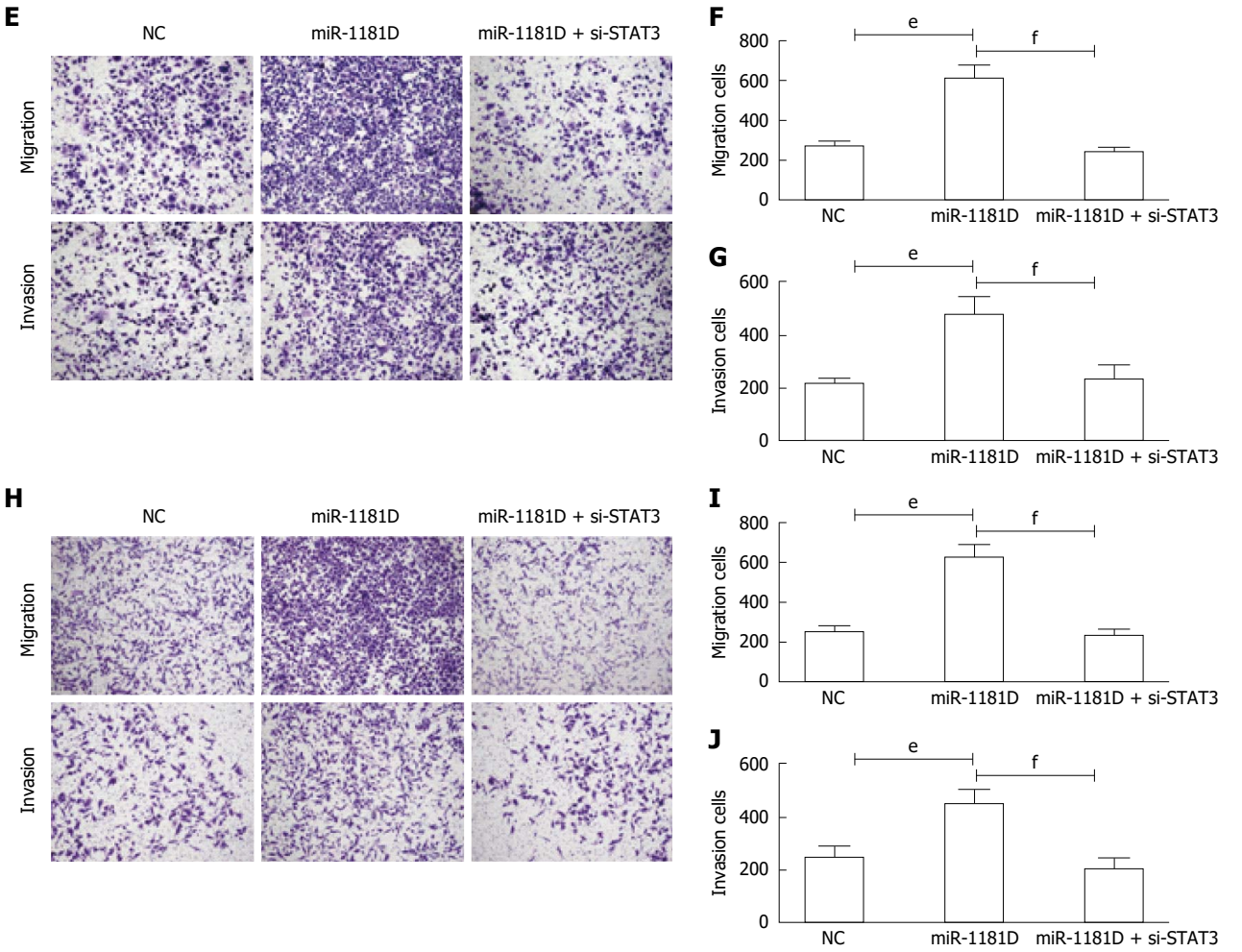


Figure 4 miR-1181 inhibits invasion and proliferation *via* STAT3 in pancreatic cancer cells. A: Indicated cells were subjected to Western blot assay to detect STAT3 and p-STAT3 expression; B: We designed 3 siRNA targeted STAT3 and verified by PCR, $^{a}P < 0.05$; C: Indicated PANC-1 cells were subjected to CCK-8 assays, $^{c}P < 0.05$; D: Indicated MIA-PaCa-2 cells were subjected to CCK-8 assays, $^{a}P < 0.05$; E: Indicated PANC-1 cells were subjected to transwell assays and relative migration and invasion capability was quantitative analyzed (F and G), $^{e}P < 0.001$, $^{f}P < 0.001$; H: Indicated MIA-PaCa-2 cells were subjected to transwell assays and relative migration and invasion capability was quantitative analyzed (I and J), $^{e}P < 0.001$, $^{f}P < 0.001$.

for various aspects of neoplasia, including proliferation, drug resistance, and survival of cancer cells through constitutive phosphorylation of STAT3[18-20]. Although numerous STAT3 inhibitors have been developed, and several STAT3 inhibitors have completed Phase Ⅰ/Ⅱ clinical trials, targeting STAT3 as a cancer therapy remains frustratingly elusive compared to targeting RTKs[18,21,22]. Numerous inhibitors for specific targets in signaling pathways have been applied in clinical use, but the success of these targeted cancer therapies has been limited by the development of resistance to these inhibitors. Therefore, there is an urgent demand to reassess the ongoing strategies to develop clinically useful drugs.

We previously found that miR-1181 inhibited stemness and tumorigenicity *via* targeting STAT3 and SOX2. Another study demonstrated that miR-1181 promoted mesenchymal-epithelial transition in ovarian cancer cells and its expression was significantly downregulated by ARK5[22].

In conclusion, here we found that miR-1181 may be involved in pancreatic cancer cell invasion and proliferation by targeting STAT3. Our findings suggest that miR-1181 may be a potential therapeutic agent for pancreatic cancer.

COMMENTS

Background

MicroRNA (miRNA) dysregulation is implicated in the development and progression of nearly all tumor types. Recent evidence has indicated that some miRNAs can function as tumor suppressors. The authors thus investigated whether miR-1181 suppressed the invasion and proliferation of pancreatic cancer.

Research frontiers

The authors previously found that miR-1181 plays an important role in inhibiting stemness and tumorigenicity of pancreatic cancer stem cells *via* targeting multiple key cancer stem cell regulators, including STAT3 and SOX2. And miR-1181 promoted mesenchymal-epithelial transition in ovarian cancer cells and its expression was significantly downregulated by ARK5.

Innovations and breakthroughs

The authors found that miR-1181 may be involved in pancreatic cancer cell

invasion and proliferation by targeting STAT3.

Applications

These findings suggest that miR-1181 may be a potential therapeutic agent for pancreatic cancer.

Terminology

miRNAs are noncoding 17- to 25-nucleotide-long RNAs that posttranscriptionally regulate the expression of multiple genes. miRNA dysregulation is implicated in the development and progression of nearly all tumor types. Recent evidence has indicated that some miRNAs can function as tumor suppressors.

Peer-review

The manuscript by Wang and co-workers analyzes the effects of miR-1181 on invasion and proliferation of pancreatic cancer cells with special emphasis on STAT-3. The manuscript is well written and the experimental procedure are sound and valid.

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ORIGINAL ARTICLE

Case Control Study

Association between *TLR7* copy number variations and hepatitis B virus infection outcome in Chinese

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Author contributions: Li F, Gao YF and Ye J contributed to the literature search and the writing of the manuscript; Li X and Zou GZ contributed to the original idea, as well as the polishing and final proof of the manuscript.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at aaylixu@qq.com. Participants gave informed consent for data sharing.

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Abstract

AIM

To explore whether copy number variations (CNVs) of toll-like receptor 7 (*TLR7*) are associated with susceptibility to chronic hepatitis B virus (HBV) infection.

METHODS

This study included 623 patients (495 males and 128 females) with chronic hepatitis B virus infection (CHB) and 300 patients (135 females and 165 males) with acute hepatitis B virus infection (AHB) as controls. All CHB patients were further categorized according to disease progression after HBV infection (CHB, liver cirrhosis, or hepatocellular carcinoma). Copy numbers of the *TLR7* gene were measured using the AccuCopy method. χ^2 tests were used to evaluate the association between *TLR7* CNVs and infection type. *P* values, odds ratios, and 95% confidence intervals (CIs) were used to estimate the effects of risk.

RESULTS

Among male patients, there were significant differences between the AHB group and CHB group in the distribution of *TLR7* CNVs. Low copy number

of *TLR7* was significantly associated with chronic HBV infection (OR = 0.329, 95%CI: 0.229-0.473, $P < 0.001$). Difference in *TLR7* copy number was also found between AHB and CHB female patients, with low copy number again associated with an increased risk of chronic HBV infection (OR = 0.292, 95%CI: 0.173-0.492, $P < 0.001$). However, there were no significant differences in *TLR7* copy number among the three types of chronic HBV infection (CHB, liver cirrhosis, or hepatocellular carcinoma). In addition, there was no association between TLR7 copy number and titer of the HBV e antigen.

CONCLUSION

Low *TLR7* copy number is a risk factor for chronic HBV infection but is not associated with later stages of disease progression.

Key words: Toll-like receptor 7; Hepatitis B virus; Copy number variations; Gene susceptibility

Core tip: Differences in patient genetic backgrounds may influence the quality of the immune response and may result in different hepatitis B virus (HBV) infection outcomes. Toll-like receptor 7 (*TLR7*) is involved in the sensing of viruses and priming of the subsequent immune response. We investigated the association between copy number at the *TLR7* locus and genetic susceptibility to chronic HBV infection. Comparison of individuals with chronic and acute HBV revealed that low *TLR7* copy number was associated with chronic but not acute HBV in both males and females, though it was not associated with subsequent disease progression.

Li F, Li X, Zou GZ, Gao YF, Ye J. Association between *TLR7* copy number variations and hepatitis B virus infection outcome in Chinese. *World J Gastroenterol* 2017; 23(9): 1602-1607 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1602.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1602

INTRODUCTION

The outcome of hepatitis B virus (HBV) infection is influenced by not only the virus but also the host's immune response and genetic diversity, including single nucleotide polymorphism (SNPs) and gene copy number variants (CNVs)[1-3]. Many studies support the idea that host genetic susceptibility plays an important role in determining the outcome of HBV infection[4]. Previous studies have shown that several SNP sites are associated with the development of chronic HBV infection and the progression of disease[5-7].

CNVs arise when individuals carry different numbers of copies of the same DNA sequence. These may be the result of deletions or duplications and insertions, and they typically cover about 15% of the human genome in each individual. CNVs are a common source of variation in the human genome and are known to play an important role in complex genetic diseases. In addition to affecting the copy number (CN) of a DNA sequence[8,9], CNVs may affect gene expression through dosage and position effects, which may further influence an individual's disease susceptibility and progression[9-11]. In humans, CNVs have so far been identified as a hereditable source of susceptibility to complex diseases such as systemic lupus erythematosus (SLE), type 1 diabetes, HBV infection, and Alzheimer's disease[8,12].

Toll-like receptors (TLRs) function as critical pattern recognition receptors (PRRs). TLRs recognize foreign pathogens as well as necrosis of endogenous substances, and play a role in both the innate and acquired immune responses[13-15]. TLRs are widely expressed in various tissues, with individual receptors exhibiting specific cell distributions. *TLR7* is a highly conserved gene on the X chromosome[16,17], and its product is expressed primarily in dendritic cells (pDC), B cells, and macrophages. TLR7 recognizes single-stranded RNA through the MyD88-dependent signaling pathway, inducing pro-inflammatory factors and the production of type I interferon to mediate the body's non-specific immune response. Activation of TLR7 initiates downstream signaling cascades through transcription factors such as interferon regulatory factor 7. This induces production of pro-inflammatory cytokines and chemokines, which are involved in various HBV infection outcomes, including spontaneous clearance and viral persistence. The role of *TLR7* CNVs in the outcome of HBV infection has not yet been investigated. In the present study, we categorized the groups according to gender, analyzed the *TLR7* CNVs, and found that there is indeed an association between the CN of the *TLR7* gene and genetic susceptibility to chronic HBV infection.

MATERIALS AND METHODS

Subjects

A total of 923 Han Chinese individuals with HBV infection were enrolled in this study from 2010 to 2015. Following recruitment, all subjects gave informed consent for genetic analysis. Among the patients, 623 (495 males and 128 females) suffered from chronic HBV (CHB) infection. The remaining 300 (135 females and 165 males) were individuals with acute, self-limiting HBV (AHB) clearance and served as the control group. Average ages were 44.41 ± 15.32 years in the CHB group and 45.26 ± 15.54 years in the AHB group. All CHB patients fulfilled the diagnostic criteria of being positive for hepatitis B surface antigen (HBsAg) for a period of at least 6 mo, with serum HBV DNA level > 1000 copies/mL. AHB patients were positive

Table 1 Forward and reverse primers of *TLR7* target segments

Probe	Chromosome	Location (ref 37 database)[1]	Amplification length (sample, competitive)[2]	Primer binding region 1	Primer binding region 2
TLR7-2	ChrX	12885611-12885868	285 (+0, -2)	GGGCCCATCTCAAGCTGATCT	TAATGAAGGGGGCATGTCACAA
TLR7-3	ChrX	12905987-12906102	143 (+0, -2)	GCACCTGTGATGCTGTGTGGTT	AGCACACAAGGGCCAAAGTGTG

[1]GRCH37 primary reference assembly; [2]Sample: Sample DNA; Competitive: Competitive DNA.

Table 2 Baseline characteristics of study patients

	n	Males/females	Age (yr)	95%CI
AHB	300	165/135	45.26 ± 15.54	43.94-47.03
CHB	253	198/55	33.98 ± 12.07	32.49-35.48
LC	248	196/52	50.26 ± 13.29	48.60-51.92
HCC	122	101/21	53.72 ± 12.50	51.48-55.96

Comparison of age among groups: F = 83.216, $P < 0.01$; comparison of sex among groups: $\chi^2 = 60.296$, $P < 0.01$. AHB: Acute hepatitis B virus infection; CHB: Chronic hepatitis B virus infection; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

for hepatitis B surface antibody and hepatitis B core antibody but negative for HBsAg, and had no history of HBV vaccination. AHB controls were age-matched with CHB cases ($t = 0.823$, $P > 0.05$). The 623 CHB patients were further classified according to disease progression: 253 patients had CHB only, 248 had liver cirrhosis (LC), and 122 had hepatocellular carcinoma (HCC). None of these patients had received anti-HBV therapy or had overlapping infections of hepatitis A, C, D, E, or G. CHB patients were also free from drug-induced hepatitis, alcoholic liver disease, fatty liver disease, and pregnancy. All of them have given consent for this study.

DNA extraction

Peripheral blood was collected from all subjects in vacuum blood tubes containing EDTA-K2. Genomic DNA was isolated from 2 mL whole blood using a Qiagen kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and was stored at -20 ℃ before CNV detection.

Quantification of copy number at the TLR7 *locus using AccuCopy assay*

CNs of the *TLR7* gene were measured using the AccuCopy method (Genesky Biotechnologies Inc., Shanghai, China). The basic molecular principle of AccuCopy's competitive PCR amplification was illustrated by Du *et al*[18]. Forward and reverse primers of target segments are listed in Table 1.

Competitive DNAs for the two references and 12 target segments were designed and synthesized as double-stranded DNA by Genesky Biotechnologies. Sequences of synthesized competitive DNAs were identical to their reference sequences in the human reference genome except for an introduced 2-bp deletion. The synthesized competitive DNAs for target and reference segments were first mixed with a defined amount of genomic DNA from each patient and then subjected to multiplex fluorescence competitive PCR amplification, which simultaneously amplified all reference and target segments from both the sample DNA and competitive DNA using multiple fluorescence-labeled primer pairs.

Thermal cycling conditions for multiplex competitive PCR amplification were based on the manufacturer's instructions. PCR products were diluted 20-fold before being loaded on to an ABI 3130XL (Applied Biosystems, Carlsbad, CA, United States) genetic analyzer for capillary electrophoresis. Raw data were analyzed by GeneMapper 4.0 (Applied Biosystems), and height data for all specific peaks were exported into a Microsoft Excel file where the peak ratio of sample DNA to competitive DNA (S/C) for each segment was calculated. After normalization using the reference segment's peak ratio, the CN ratio of each target segment to the reference was determined by the target's peak ratio divided by the reference's peak ratio.

Statistical analysis

The distributions of *TLR7* CNs were compared between CHB patients and AHB control subjects using χ^2 test. *P* values, odds ratios (ORs), and 95% confidence intervals (CIs) were estimated using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, United States).

RESULTS

Sample demographics

TLR7 gene CNs were analyzed and quantified in triplicate in 623 Chinese CHB patients and 300 Chinese AHB patients. Baseline patient characteristics are shown in Table 2. While there were no significant age differences between AHB and CHB patients (including CHB, LC and HCC groups) ($t = 0.823$, $P = 0.944 > 0.05$), there was a difference if the CHB group was divided into CHB, LC and HCC groups (F = 83.216, $P < 0.01$).

Frequency distribution of TLR7 *gene copy number*

A reference category was chosen based on the median *TLR7* CN obtained in the female and male control groups (CN = 2 for females and CN = 1 for males), as previously described[19]. Based on the reference category, the risk of disease progression associated

Table 3 Estimated risk of *TLR7* copy number in the development of chronic hepatitis B virus infection

Gender	CN	Chronic group	Acute group	OR	95%CI:	*P* value
Male	< 1	337	68	0.33	0.23-0.47	< 0.001
	> 1	158	97			
Female	< 2	96	63	0.29	0.17-0.49	< 0.001
	> 2	32	72			

CN: Copy number.

Table 4 Frequency distribution of *TLR7* copy number in chronic hepatitis B virus infection groups

Gender	CN	CHB	LC	HCC	χ^2	*P* value
Male	< 1	137	137	63	1.923	0.382
	> 1	61	59	38		
Female	< 2	39	42	15	1.557	0.459
	> 2	16	10	6		

CN: Copy number; CHB: Chronic hepatitis B virus infection; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

with the absolute *TLR7* CN was estimated by comparing cases and controls that were categorized as CN > 2 or CN ⩽ 2 for females and as CN > 1 or CN ⩽ 1 for males. The frequency distributions of *TLR7* CNs of the different patient groups are shown in Figure 1.

We examined the single-locus association between *TLR7* CN and susceptibility to chronic HBV infection. *TLR7* CNs were significantly different between the acute and chronic group in both male and female patients (males: χ^2 = 37.682, P < 0.001; females: χ^2 = 22.063, P < 0.001). As shown in Table 3, low *TLR7* CN was significantly associated with chronic HBV infection in males (OR = 0.329, 95%CI: 0.229-0.473) and females (OR = 0.292, 95%CI: 0.173-0.492). We also compared the *TLR7* CN distributions of the CHB, LC, and HCC groups, but there were no significant differences among the three groups (Table 4). HBeAg can play a role of immune regulation which is associated with the outcome of HBV infection. E antigen was used as an important index of HBV replication and infection. We divided patients into two groups according to e-antigen titers (0-1 IU/mL *vs* > 1 IU/mL). However, titer levels did not vary significantly among patient groups (Figure 2).

DISCUSSION

The disease spectrum of HBV infection ranges from acute HBV infection to chronic HBV carrier to chronic hepatitis, liver cirrhosis, and HCC. Many studies have provided evidence to support the idea that host genetic variation and immunity play important roles in determining the outcome of HBV infection[20]. The gene for Toll-like receptor TLR7 is located on the X chromosome and so experiences sex-linked

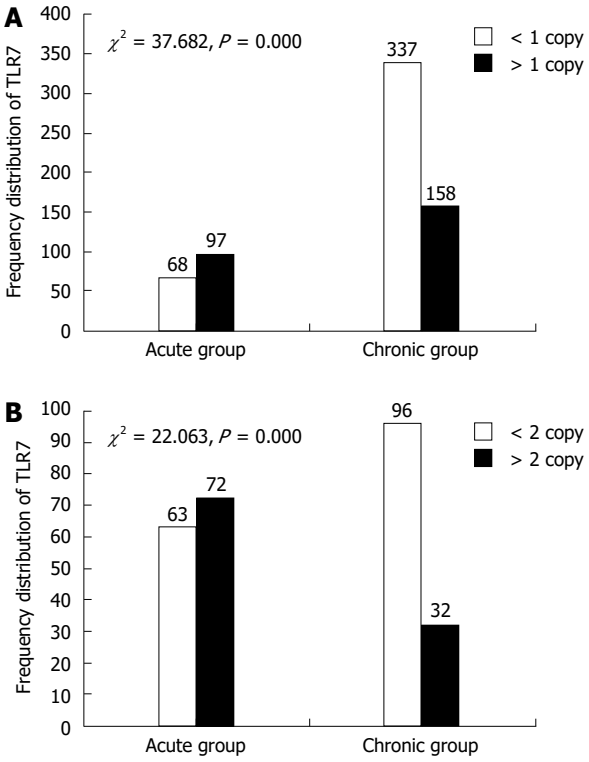

Figure 1 Frequency distribution of *TLR7* gene copy numbers in male and female patients. The chronic group includes patients with chronic hepatitis B viral hepatitis, liver cirrhosis, and hepatocellular carcinoma. A: Male patients; B: Female patients.

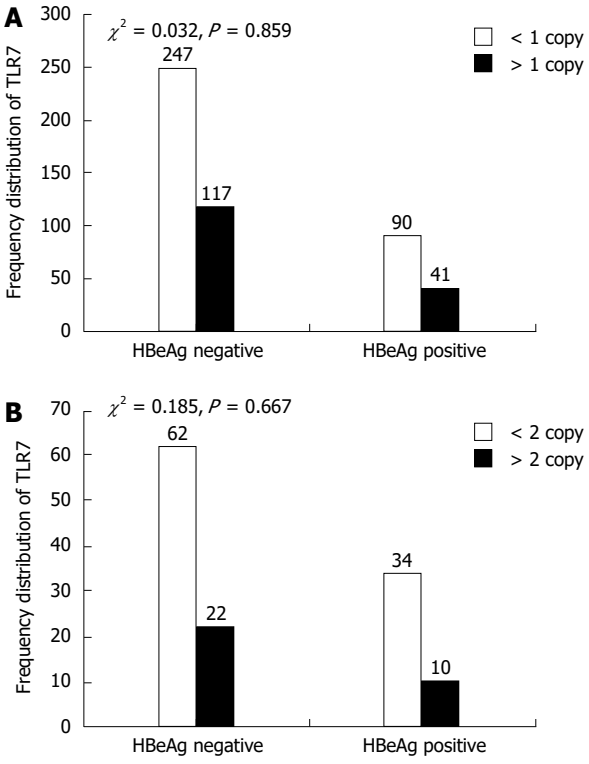

Figure 2 Frequency distribution of *TLR7* gene copy numbers in males and females according to e-antigen titer. Patients were divided into two groups according to the titer of e antigen: HBeAg negative (0-1 IU/mL) and HBeAg positive (> 1 IU/mL). A: Male patients; B: Female patients.

inheritance. Therefore, analysis of *TLR7* CNVs suggested that the data should be separated according to gender. *TLR7* CNVs have been previously found to be associated with SLE[20], while Lund *et al*[21] found that TLR7 is involved in the body's antiviral immune response. In addition, some researchers believe that TLR7 represents a new sensor of viral infections[22]. However, its role in HBV infection has not been previously determined. We therefore designed this study to explore the association between *TLR7* CNVs and chronic HBV susceptibility. In a previous study, we found that up-regulation of TLR7 is essential for serological clearance of hepatitis B surface antigen in HBV infection. The results in the current study revealed that low *TLR7* CNs were significantly associated with chronic HBV infection in both males and females, suggesting that elimination of HBsAg is more difficult with reduced levels of TLR7. This indicates that a reduction in *TLR7* CN is a risk factor for chronic HBV in Han Chinese patients. Hui *et al*[23] found that increased expression of TLR7 in the recovery phase may be involved in the clearance of HBsAg in patients with CHB. García-Ortiz *et al*[19] found that *TLR7* mRNA levels correlated significantly with *TLR7* CN, suggesting that an increase in *TLR7* gene dosage results in the up-regulation of *TLR7* mRNA expression in humans as well as in murine models of lupus. Moreover, Xu *et al*[24] found that PBMCs and pDCs from CHB patients exhibited diminished capacities to produce IFN-α in response to TLR7 activation. The fact that TLR7 is expressed in pDCs rather than in mDCs indicates that pDCs are potentially involved in fighting against viral infections. Therefore, reduced expression of TLRs in pDCs of CHB patients may lead to functional defects, as demonstrated by diminished production of IFN-α, resulting in persistent HBV infection. We also found that the replication system is adapted to generate high levels of virions without stimulating the innate immune system. Secreted viral proteins (HBsAg and HBeAg) suppress innate responses through inhibition of TLR signaling, which leads to a weak adaptive immune response with an exhausted phenotype that is incapable of inducing viral elimination[25]. Our experiment aims to study the relationship between the two indicators at a gene level. We found no association between TLR7 copy number and titer of the HBV e antigen, which implied that there was no correlation at a gene level between the two indicators.

In conclusion, this was the first study to independently analyze the relationship between *TLR7* CNVs and susceptibility to chronic HBV. Our findings indicate that low copy numbers of the *TLR7* gene may represent a risk factor for chronic HBV infection. Further replication and functional studies are necessary to confirm these results. In particular, as the biological functions of *TLR7* CNVs in HBV infection have not been fully elucidated, further research is required to determine the mechanistic basis by which *TLR7* CNVs affect the immune response.

COMMENTS

Background

More and more research suggest that the outcome of hepatitis B virus (HBV) infection is influenced by not only the virus but also the host's immune response and genetic diversity, including SNPs and gene copy number variations (CNVs). TLRs recognize foreign pathogens as well as necrosis of endogenous substances, and play a role in both the innate and acquired immune responses, but the role of Toll-like receptor 7 (TLR7) CNVs in the outcome of HBV infection has not yet been investigated.

Research frontiers

In humans, CNVs have so far been identified as a hereditable source of susceptibility to complex diseases such as systemic lupus erythematosus type 1 diabetes, HBV infection, and Alzheimer's disease. Some researchers believe that TLR7 represents a new sensor of viral infections. However, its role in HBV infection has not been previously determined.

Innovations and breakthroughs

In this study, the authors found that there were significant differences between the acute patients and chronic patients in TLR7 copy number, with low copy numbers of TLR7 associated with chronic HBV infection in both males and females. This study identified a significant risk factor for chronic hepatitis B infection.

Applications

This study sheds light on the major underlying mechanisms of hepatitis B, which may have future clinical implications.

Peer-review

This is a really interesting study since TLR7 is involved in outcome of viral infections such as hepatitis C virus, HBV or human immunodeficiency virus, but no evidence is available about the association of CNV in TLR7 and HBV. The authors compared TLR7 CNVs in patients with acute HBV infection and chronic infection and among patients with chronic HBV classified according to disease progression.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Mortality associated with gastrointestinal bleeding in children: A retrospective cohort study

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Abstract

AIM

To determine the clinical characteristics of children with gastrointestinal bleeding (GIB) who died during the course of their admission.

METHODS

We interrogated the Pediatric Hospital Information System database, including International Classification of Diseases, Current Procedural Terminology and Clinical Transaction Classification coding from 47 pediatric tertiary centers extracting the population of patients (1-21 years of age) admitted (inpatient or observation) with acute, upper or indeterminate GIB (1/2007-9/2015). Descriptive statistics, unadjusted univariate and adjusted multivariate analysis of the associations between patient characteristics and treatment course with mortality was performed with mortality as primary and endoscopy a secondary outcome of interest. All analyses were performed using the R statistical package, v.3.2.3.

RESULTS

The population with GIB was 19528; 54.6% were male, overall mortality was 2.07%; (0.37% in patients with the principal diagnosis of GIB). When considering

only the mortalities in which GIB was the principal diagnosis, 48% (12 of 25 principal diagnosis GIB mortalities) died within the first 3 d of admission, whereas 19.8% of secondary diagnosis GIB patients died with 3 d of admission. Patients who died were more likely to have received octreotide (19.8% *c.f.* 4.04%) but tended to have not received proton pump inhibitor therapy in the first 48 h, and far less likely to have undergone endoscopy during their admission (OR = 0.489, $P < 0.0001$). Chronic liver disease associated with a greater likelihood of endoscopy. Mortalities were significantly more likely to have multiple complex chronic conditions.

CONCLUSION

GIB associated mortality in children is highest within 7 d of admission. Multiple comorbidities are a risk factor whereas early endoscopy during the admission is protective.

Key words: Pediatrics; Gastrointestinal hemorrhage; Endoscopy; Proton pump inhibitors; Mortality; Liver disease; Hospital Information Systems; Octreotide

Core tip: The management of gastrointestinal haemorrhage in children is challenging insofar as the timing and impact of different interventions remains poorly defined. The authors analysed the characteristics and associated interventions associated with mortality as an outcome with gastrointestinial bleeding in children past infancy. Death associated with gastrointestinal haemorrhage was reported in 2% overall albeit less (0.4%) in the cohort with haemorrhage as admitting diagnosis. Patients who died were far less likely to have undergone endoscopy during the admission and more likely to have received octreotide and less likely to have received proton pump inhibitor therapy during the first two days of admission.

Attard TM, Miller M, Pant C, Kumar A, Thomson M. Mortality associated with gastrointestinal bleeding in children: A retrospective cohort study. *World J Gastroenterol* 2017; 23(9): 1608-1617 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1608.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1608

INTRODUCTION

Gastrointestinal bleeding (GIB) is a foremost indication for emergent diagnostic and therapeutic endoscopy requiring prompt, including disease-specific pharmacotherapy[1,2]. Although acute GIB in adults has been exhaustively studied including epidemiology and predictors of adverse outcomes, there is a paucity of the corresponding evidence in children[3,4]. This deficit hinders the evidence based allocation of resources and the implementation of standardized protocols, potentially adversely impacting outcomes in children.

One of the co-authors, has identified the presence of > 3 comorbid conditions, presentation to a teaching hospital, the presence of upper GIB; age under 5 years and health coverage with private insurance as independent risk factors associated with an increased rate of hospital admission with GIB[5]. Hemorrhage occurred in 0.5% of all discharges from inpatient care, was more prevalent in males and older than 11 years. Esophageal and intestinal perforation were identified as at highest risk of associated mortality, together accounting for 17% of all patients with GI haemorrhage and who died[6].

Disease classification in adult GIB cannot be extrapolated to the pediatric population. Risk factors identifiable in adults, foremost amongst which are age, non-steroidal anti-inflammatory drug, selective serotonin reuptake inhibitor, aspirin, antiplatelet and anticoagulant therapy and chronic renal and cardiovascular disease, are clearly not applicable to children[7]. Conversely, the impact of predominantly pediatric and especially neonatal disease processes (*e.g.,* prematurity) on the risk of GIB remain unknown. This limits the applicability of pre- and postendoscopic predictive scoring systems [Rockall, Blatchford (aka Glasgow), Addenbrooke] to identify patients at high risk (need for blood transfusion, surgical intervention, rebleeding and mortality) and those requiring immediate endoscopic intervention as opposed to at low risk who can be safely discharged[8-10]. The Sheffield Scoring system is, to date the only successful attempt at predicting the need for endoscopic hemostatic intervention based on the clinical presentation, hemodynamic parameters and need for blood products[11]. An understanding of the epidemiologic context of GIB in children holds the promise of directing future research toward improving predictive models of disease outcomes including mortality.

The Pediatric Health Information System (PHIS) database is a repository of diagnostic, therapeutic and procedure records from 48 regional pediatric tertiary centers in the United States that has been in existence since 2004, the data is available in de-identified form to health information management administrators and academicians in the respective institutions.

Herein we report on the PHIS recorded demographic and clinical profile of children with upper or indeterminate gastrointestinal bleeding at admission or during their inpatient course and resulting in death.

MATERIALS AND METHODS

Data source

We conducted a retrospective cohort study using data obtained from the PHIS, an administrative database that contains inpatient, emergency department,

ambulatory surgery and observation encounter-level data from 49 not-for-profit, tertiary care pediatric hospitals in the United States. The PHIS hospitals are 49 of the largest and most advanced children's hospitals in America, and constitute the most demanding standards of pediatric service in America. These hospitals are affiliated with the Children's Hospital Association (Overland Park, KS, United States). Data quality and reliability are assured through a joint effort between the Children's Hospital Association and participating hospitals. Portions of the data submission and data quality processes for the PHIS database are managed by Truven Health Analytics (Ann Arbor, MI, United States). For the purposes of external benchmarking, participating hospitals provide discharge/encounter data including demographics, diagnoses, and procedures. Nearly all of these hospitals also submit resource utilization data (*e.g.,* pharmaceuticals, imaging, and laboratory) into PHIS. Data are de-identified at the time of data submission, and data are subjected to a number of reliability and validity checks before being included in the database. For this study, data from 47 hospitals was included. This study was approved by the Institutional Review Board (16050358).

Study patients

Children between the ages of 1 and 21 years at the time of admission were eligible for inclusion if they were diagnosed with an upper gastrointestinal bleed (UGIB) or GIB of indeterminate location and admitted as an inpatient or under observation with Emergency Department charges between January 1, 2007 and September 30, 2015. Study participants with UGIB were identified through International Classification of Diseases, Ninth Revision (ICD-9) discharge diagnosis codes (Supplementary Table 1).

Demographic characteristics included age in years at time of admission, sex, race/ethnicity (non-Hispanic White, non-Hispanic Black, Hispanic, Asian, Other, Unknown), discharge disposition (routine/home, expired and rural *vs* urban zip code of residence. Complex chronic conditions (CCCs) were defined using a previously described ICD-9 coding scheme for 9 types of CCCs (neuromuscular, cardiovascular, respiratory, renal, gastrointestinal, hematologic/immunologic, metabolic, congenital or genetic, and malignancy), as well as organ transplant patients and technology dependent patients[12]. A given patient could have could have more than 1 CCC, and the total number of CCCs for each patient was calculated. Chronic liver disease was also identified by ICD-9 diagnosis codes, and coded as a dichotomous variable. The need for packed red blood cell transfusions was used to control for severity of bleeding [0 = no transfusion, 1 = transfusion(s) received].

Procedures were identified through ICD-9-CM codes (Supplementary Table 2), and pharmaceuticals and imaging procedures were identified through Clinical Transaction Classification System for revenue codes.

Outcome measures

The primary outcome of interest in this study was mortality. Secondary outcomes examined include whether or not the patient underwent endoscopy.

Statistical analysis

Unadjusted, univariate analyses of the associations between patient characteristics and treatment course with mortality were carried out. Continuous variables were summarized using the median and interquartile ranges (IQR) and compared using the Wilcoxon rank-sum test. Categorical variables were summarized using counts and frequency as a percentage, and compared using the χ^2 test of association or Fisher's exact test, where appropriate. Complex chronic conditions were treated categorically as the number of complex chronic conditions present in a single patient. The levels of the category were defined as 0 complex chronic conditions, 1 or 2 complex chronic conditions, and 3 or more complex chronic conditions. These levels were chosen after assessing the median and interquartile range of the distribution of number of CCCs. Receipt of pharmaceuticals on the first or second day of admission was coded as a dichotomous variable, as was the receipt of packed red blood cell transfusions and platelet transfusions. All procedures were coded as 0 (procedure not billed) or 1 (procedure billed). Unadjusted *P*-values were reported for the univariate analysis.

Adjusted analysis of the association between patient characteristics and treatment course with mortality were examined using multivariable generalized linear mixed models to assess the odds of exposure to treatment among mortality cases (binomial family, logit link). A quasi-likelihood method was used to estimate effects (Laplace approximation). All candidate models were adjusted for potential confounding by age in years at admission, race/ethnicity, and sex and by the need for packed red blood cell transfusions as a surrogate of severity of bleed. Chronic liver disease, comorbid complex chronic conditions, and urban *vs* rural zip code of residence at time of admission were tested as covariates. Other covariates included perforation type injury, administration of proton pump inhibitor (PPI), H_2RA, octreotide, and vasopressin pharmaceuticals on the first or second day of admission and endoscopic procedures performed. Interactions between vasopressin and shock, endoscopy and chronic liver disease, and endoscopy and CCCs were also tested to investigate potential effect modification. To account for increased variability due to clustering within hospitals, a random intercept was included using a unique hospital ID. Model selection was carried out using -2 log likelihood tests with the χ^2 approximation. Individual covariates were tested by approximation to the *Z*-value. The Holm procedure was used to account

Table 1 Patient characteristics by principal and secondary diagnosis of gastrointestinal bleeding *n* (%)

	GI bleed		Overall (*n* = 19528)	*P* value
	Principal Dx (*n* = 6733)	Secondary Dx (*n* = 12795)		
Age in years (IQR)	10 (4, 15)	9 (4, 15)	9 (4, 15)	< 0.0001
Gender				< 0.0001
Female	2925 (43.44)	5941 (46.43)	8866 (45.40)	
Male	3808 (56.56)	6854 (53.57)	10762 (55.11)	
Race				< 0.0001
Non-Hispanic White	3608 (53.59)	6596 (51.55)	10204 (52.25)	
Non-Hispanic Black	1180 (17.53)	2257 (17.64)	3437 (17.60)	
Hispanic	1159 (17.21)	2580 (20.16)	3739 (19.15)	
Asian	251 (3.73)	332 (2.59)	583 (2.99)	
Other	412 (6.12)	779 (6.09)	1191 (6.10)	
Unknown	123 (1.83)	251 (1.96)	374 (1.92)	
Urban/rural				0.1247
Urban	5730 (85.10)	10930 (85.42)	16660 (85.31)	
Rural	873 (12.97)	1573 (12.29)	2446 (12.53)	
Unknown	130 (1.93)	292 (2.28)	422 (2.16)	
Complex chronic conditions				< 0.0001
0	3767 (55.95)	5969 (46.65)	9736 (49.86)	
1-2	1771 (26.30)	4328 (33.83)	6099 (31.23)	
⩾ 3	1195 (17.75)	2498 (19.52)	3693 (18.91)	
GIH symptoms				
Hematemesis	2333 (34.65)	4263 (33.32)	6596 (33.78)	0.0635
Melena	1983 (29.45)	6018 (47.03)	8001 (40.97)	< 0.0001
Hypovolemia	52 (0.77)	90 (0.70)	142 (0.73)	0.5956
Pharmaceutical Interventions				
PPI on day 0 or 1	4473 (66.43)	6038 (47.19)	10511 (53.83)	< 0.0001
H_2RA on day 0 or 1	1301 (19.32)	2715 (21.22)	4016 (20.57)	0.0019
Erythromycin on day 0 or 1	197 (2.93)	313 (2.45)	510 (2.61)	0.0511
Vasopressin on day 0 or 1	15 (0.22)	142 (1.11)	157 (0.80)	< 0.0001
Octreotide on day 0 or 1	349 (5.18)	439 (3.43)	788 (4.04)	< 0.0001
Diagnostic Imaging				
Meckel's Scan day 0-2	497 (7.38)	378 (2.95)	875 (4.48)	< 0.0001
Abdomen CT day 0-2	425 (6.31)	1298 (10.14)	1723 (8.82)	< 0.0001
Abdomen MRI day 0-2	44 (0.65)	170 (1.33)	214 (1.10)	< 0.0001
Arteriography day 0-2	75 (1.11)	93 (0.73)	168 (0.86)	0.0070
Surgical interventions				
Laparotomy, exploratory	23 (0.34)	44 (0.34)	67 (0.34)	0.9999
Laparotomy, other	2 (0.03)	19 (0.15)	21 (0.11)	0.0191
Laparoscopy	54 (0.80)	96 (0.75)	150 (0.77)	0.7303
EGD	2304 (34.22)	2317 (18.11)	4621 (23.66)	< 0.0001
Other endoscopy	845 (12.55)	845 (6.6)	1690 (8.65)	< 0.0001
Transcatheter embolization	8 (0.12)	8 (0.06)	16 (0.08)	0.1977
Ligation, esophag. varices	0 (0.0)	5 (0.04)	5 (0.03)	0.1719
Ligation, gastric varices	1 (0.01)	5 (0.04)	6 (0.03)	0.6713
Packed red blood cell transfusions	1265 (18.79)	2337 (18.26)	3602 (18.45)	0.3808
Platelet transfusions	194 (2.88)	799 (6.24)	993 (5.09)	< 0.0010
ICU stay as part of encounter	880 (13.07)	2328 (18.19)	3208 (16.43)	< 0.0001
Chronic liver disease	171 (2.54)	400 (3.13)	571 (2.92)	0.0234
GIH present on admit	4980 (73.96)	8585 (67.10)	11084 (56.76%)	< 0.0001
Shock	144 (2.14)	584 (4.56)	728 (3.73)	< 0.0001
Sepsis	30 (0.45)	666 (5.21)	696 (3.56)	< 0.0001
Hospital lOS (IQR)	2 (1, 4)	3 (2, 7)	3 (1, 6)	< 0.0001
Day of EGD	1 (0, 2)	2 (1, 3)	1 (0, 2)	< 0.0001
Mortality	25 (0.37)	379 (2.96)	404 (2.07)	< 0.0001

IQR: Interquartile ranges; GER: Gastroesophageal reflux; PPI: Proton pump inhibitor; H_2RA: H_2-receptor antagonists; CT: Computed tomography; MRI: Magnetic resonance imaging; GIB: Gastrointestinal bleeding; EGD: Esophagogastroduodenoscopy.

for multiple testing of covariates for each outcome, and the adjusted *P*-values are reported for the significance test of model covariates. An adjusted, two-tailed $P < 0.05$ was considered statistically significant. All analyses were performed using the R statistical package, v.3.2.3.

RESULTS

Descriptive statistics

There were 19528 patients with upper or indeterminate GIB discharged between January 1, 2007 and September 30, 2015 (Table 1). Overall, 54.6% of

Table 2 Complex chronic conditions by discharge disposition *n* (%)

	Survived (*n* = 19124)	Died (*n* = 404)	*P* value
Gastrointestinal flag	5351 (27.98)	159 (39.36)	< 0.0001
Cardiovascular flag	1543 (8.07)	162 (40.10)	< 0.0001
Hem/immunologic flag	1662 (8.69)	152 (37.62)	< 0.0001
Malignancy	1164 (6.09)	135 (33.42)	< 0.0001
Metabolic flag	1138 (5.95)	135 (33.42)	< 0.0001
Neurologic/neuromusc flag	2570 (13.44)	145 (35.89)	< 0.0001
Congenital/genetic flag	1838 (9.61)	81 (20.05)	< 0.0001
Renal/urologic flag	927 (4.85)	133 (32.92)	< 0.0001
Respiratory flag	780 (4.08)	65 (16.09)	< 0.0001
Technology depend. flag	3710 (19.40)	242 (59.90)	< 0.0001
Transplant flag	802 (4.19)	94 (23.27)	< 0.0001

patients were male, and the median age was 9 years (IQR 4-15). Nearly half (49.68%) of the patients had no documented CCCs, 30.32% had 1 or 2 CCCs, and 20.01% had 3 or more CCCs. The most common CCC was gastrointestinal conditions (28.22%), followed by technology dependence[12,13] (20.24%) and neurologic and neuromuscular disorders (13.90%) (Table 2). Of the patients included in the analysis, 33.78% experienced hematemesis, 40.97% melena, 12.18% had gastroesophageal reflux (GER), 3.73% experienced shock, 3.56% experienced sepsis, and 18.45% required packed red blood cell transfusion while 5.09% required a platelet transfusion. Most patients resided in an urban area (85.31%), although a small portion of the data was missing (2.16%).

Overall mortality rate was 2.07% with 0.37% mortality among patients with principal diagnosis of GIB and 2.96% among patients with secondary diagnosis of GIB. The median time until death was 19 d (5, 49). For all patients, 21.53% of deaths occurred within the first 3 d of admission, and 31.9% occurred within 7 d of admission. When considering only the mortalities in which GIB was the principal diagnosis, 48% (12 of 25 principal diagnosis GIB mortalities) died within the first 3 d of admission, and 64% expired within 7 d of admission. Among secondary diagnosis patients, 19.8% died with 3 d of admission, and 29.8% died within 7 d of admission. Although the majority of patients were male, females and males had similar mortality rates (50% of mortalities were male). There were apparent racial/ethnic distributional differences, with non-Hispanic Whites being the only group that comprised a smaller proportion of the mortalities than the surviving cases.

Early intervention with pharmaceuticals was more frequent among mortality cases (Table 3). Receipt of PPI on the first or second day of admission occurred in 53.83% of patients, with higher usage among mortalities (68.56% *vs* 53.31%). H_2RA were administered on the first or second day of admission in 20.57% of patients, with higher usage in mortality than non-mortality cases (37.62% *vs* 20.20%). Octreotide was only used in 4.04% of patients; 19.80% of patients who died received octreotide on the first or second day of admission, and 3.70% surviving cases received octreotide. Vasopressin was only given to 0.80% of patients; 24.50% of the mortality cases and 0.30% of surviving cases received vasopressin on the first or second day of admission.

Table 4 displays the top admitting diagnosis codes for mortalities and non-mortalities. Mortalities included several diagnosis codes not related to GI symptoms, including dyspnea and respiratory abnormalities, cardiac arrest, pneumonia, diseases of white blood cells, and respiratory failure. Non-mortalities more frequently carried GI-specific admitting diagnoses.

Multivariable analysis

Factors associated with mortality in all GIB diagnoses: After adjustment for other covariates, race was not significantly associated with mortality in patients with a primary diagnosis of UGIB or unspecified GIB (adjusted $P = 0.999$). Although the majority of patients were male, mortality tended to be higher in females; however, gender was not significantly associated with mortality (adjusted $P = 0.339$). Age was also not significantly associated with mortality (adjusted $P = 0.999$). These covariates were retained in the model for their role as potential confounders. Urban *vs* rural zip code of residence, H_2RA within the first or second day of admission, and the interaction between endoscopy and chronic liver disease were not statistically significant and did not improve fit, thus were removed from the model [$\chi^2(4) = 4.505$, $P = 0.342$]. Furthermore, chronic liver disease, and the interaction between endoscopy and CCCs were not significant and did not significantly improve model fit and were also removed from the final model [$\chi^2(3) = 4.612$, $P = 0.203$]. Although PPI on the first or second day of the encounter was not significant after correcting for multiple testing, the inclusion of this variable significantly improved model fit [$\chi^2(1) = 5.451$, $P = 0.020$].

Those patients who died were far less likely to have undergone an endoscopic procedure (OR = 0.489, 95%CI: 0.356-0.672, $P < 0.0001$), indicating a

Table 3 Univariate analysis of factors affecting mortality *n* (%)

	Survived (*n* = 19124)	Died (*n* = 404)	Overall (*n* = 19528)	*P* value
Age in years (IQR)	9 (4, 15)	8 (3, 15)	9 (4, 15)	0.2997
Gender				0.0679
Female	8864 (46.35)	202 (50)	9066 (46.43)	
Male	10460 (54.70)	202 (50)	10662 (54.60)	
Race				0.0005
Non-Hispanic White	10036 (52.48)	168 (41.58)	10204 (52.25)	
Non-Hispanic Black	3358 (17.56)	79 (19.55)	3437 (17.60)	
Hispanic	3646 (19.07)	93 (23.02)	3739 (19.15)	
Asian	565 (2.95)	18 (4.46)	583 (2.99)	
Other	1156 (6.04)	35 (8.66)	1191 (6.10)	
Unknown	363 (1.90)	11 (2.72)	374 (1.92)	
Urban/rural				0.2524
Urban	16324 (85.36)	336 (83.17)	16660 (85.31)	
Rural	2385 (12.47)	61 (15.10)	2446 (12.53)	
Unknown	415 (2.17)	7 (1.73)	422 (2.16)	
Complex chronic conditions				< 0.0001
0	9689 (50.66)	12 (2.97)	9701 (49.68)	
1-2	5825 (30.46)	95 (23.51)	5920 (30.32)	
⩾ 3	3610 (18.88)	297 (73.51)	3907 (20.01)	
GIH symptoms				
Hematemesis	6508 (34.03)	88 (21.78)	6596 (33.78)	< 0.0001
Melena	7899 (41.30)	102 (25.25)	8001 (40.97)	< 0.0001
Hypovolemia	131 (0.69)	11 (2.72)	142 (0.73)	0.0002
GER	2329 (12.18)	50 (12.38)	2379 (12.18)	0.9653
Pharmaceutical interventions				
PPI first 24 h	10234 (53.51)	277 (68.56)	10511 (53.83)	< 0.0001
H_2RA first 24 h	3864 (20.20)	152 (37.62)	4016 (20.57)	< 0.0001
Erythromycin first 24 h	480 (2.51)	30 (7.43)	510 (2.61)	< 0.0001
Vasopressin first 24 h	58 (0.30)	99 (24.50)	157 (0.80)	< 0.0001
Octreotide first 24 h	708 (3.70)	80 (19.80)	788 (4.04)	< 0.0001
Surgical interventions				
Laparotomy, exploratory	54 (0.28)	13 (3.22)	67 (0.34)	< 0.0001
Laparotomy, other	16 (0.08)	5 (1.24)	21 (0.11)	< 0.0001
Laparoscopy	144 (0.75)	6 (1.49)	150 (0.77)	0.1347
EGD	4569 (23.89)	52 (12.87)	4621 (23.66)	< 0.0001
Other endoscopy	1638 (8.57)	52 (12.87)	1690 (8.65)	0.0031
Transcatheter embolization	14 (0.07)	2 (0.50)	16 (0.08)	0.0423
Ligation, esophag. varices	4 (0.02%)	1 (0.25%)	5 (0.03)	0.0993
Ligation, gastric varices	6 (0.03)	0 (0.00)	6 (0.03)	0.9999
Diagnostic imaging				
Meckel's Scan day 0-2	873 (4.56)	2 (0.50)	875 (4.48)	0.0001
Abdomen CT day 0-2	1675 (8.76)	48 (11.88)	1723 (8.82)	0.0356
Abdomen MRI day 0-2	212 (1.11)	2 (0.50)	214 (1.10)	0.3340
Arteriography day 0-2	163 (0.85)	5 (1.24)	168 (0.86)	0.4031
Packed red blood cell Transfusion	3361 (17.57)	241 (59.65)	3602 (18.45)	< 0.0001
Platelet transfusion	819 (4.28)	174 (43.07)	993 (5.09)	< 0.0001
ICU stay as part of encounter	2863 (14.97)	345 (85.40)	3208 (16.43)	< 0.0001
Chronic liver disease	536 (2.80)	35 (8.66)	571 (2.92)	< 0.0001
GIB Present on admit	13386 (70.00)	179 (44.31)	11084 (56.76)	< 0.0001
Principal Dx GIB	6708 (35.07)	25 (6.18)	6733 (34.48)	< 0.0001
Sepsis	516 (2.70)	180 (44.55)	696 (3.56)	< 0.0001
Shock	562 (2.94)	166 (41.09)	728 (3.73)	< 0.0001
Hospital LOS (IQR)	3 (1, 6)	19 (5, 49)	3 (1, 6)	< 0.0001
Day of EGD	1 (0, 2)	3 (1, 14)	1 (0, 2)	< 0.0001

IQR: Interquartile ranges; GER: Gastroesophageal reflux; PPI: Proton pump inhibitors; H_2RA: H_2-receptor antagonists; CT: Computed tomography; MRI: Magnetic resonance imaging; GIB: Gastrointestinal bleeding; EGD: Esophagogastroduodenoscopy.

protective association with endoscopy. Mortalities were also less likely to have a GIB documented as present on admission (OR = 0.464, 95%CI: 0.362-0.596, P < 0.0001), and less likely to have had the GIB as the principal diagnosis for the encounter (OR = 0.266 95%CI: 0.165-0.429, P < 0.0001). This may suggest that GIB more commonly complicates inpatient stays for patients admitted or being primarily treated for other conditions. PPI administration on the first or second day of admission tended to be protective; however, the effect was not statistically significant after correction for multiple testing (OR = 0.723, 95%CI:

Table 4 Top 10 admitting diagnoses for mortalities and non-mortalities

ICD-9 code	ICD-9 code description	*n* (%)
Mortalities		
780.60	Fever	32 (7.92)
786.09	Other dyspnea and respiratory abnormality	23 (5.69)
787.03	Vomiting alone	15 (3.71)
03.89	Unspecified septicemia	14 (3.47)
578.0	Hematemesis	13 (3.22)
427.5	Cardiac arrest	13 (3.22)
578.9	Hemorrhage of GI tract, unspecified	12 (2.97)
486.0	Pneumonia, organism unspecified	11 (2.72)
288.00	Diseases of white blood cells	10 (2.48)
518.81	Acute respiratory failure	10 (2.48)
Non-mortalities		
578.0	Hematemesis	3058 (15.99)
578.1	Blood in Stool	2750 (14.38)
578.9	Hemorrhage of GI tract, unspecified	1289 (6.74)
789.00	Other symptoms involving abdomen and pelvis	755 (3.95)
787.03	Vomiting alone	748 (3.91)
N/A	Not available or Missing	707 (3.70)
780.60	Fever	559 (2.92)
276.51	Dehydration	515 (2.69)
787.91	Diarrhea	500 (2.61)
2859	Anemia	292 (1.53)

GI: Gastrointestinal; ICD-9: International Classification of Diseases, Ninth Revision.

Table 5 Complex chronic conditions by principal and secondary diagnosis of gastrointestinal bleeding *n* (%)

	Principal Dx is GI bleed (*n* = 4521)	Secondary Dx is GI bleed (*n* = 15007)	*P* value
GI flag	1681 (24.39)	3892 (15.39)	< 0.0001
Cardiovascular flag	513 (7.44)	1192 (4.71)	< 0.0001
Hem/immunologic flag	422 (6.12)	1392 (5.5)	< 0.0001
Malignancy	284 (4.12)	1015 (4.01)	< 0.0001
Metabolic flag	258 (3.74)	1015 (4.01)	< 0.0001
Neurologic/neuromusc flag	972 (14.1)	1743 (6.89)	0.1234
Congenital/genetic flag	727 (10.55)	1192 (4.71)	0.0010
Renal/urologic flag	249 (3.61)	811 (3.21)	< 0.0001
Respiratory flag	244 (3.54)	601 (2.38)	0.0005
Technology depend flag	1353 (19.63)	2599 (10.28)	0.7331
Transplant flag	318 (4.61)	578 (2.29)	0.5374

GI: Gastrointestinal.

0.552-0.947, $P = 0.074$).

The odds of having 1 or 2 CCCs compared to 0 CCCs were 9.090 times higher for mortalities over non-mortalities (95%CI: 4.907-16.841, $P < 0.0001$), and the odds of having 3 or more CCCs compared to 0 CCCs was 27.338 (95%CI: 14.940-50.027, $P < 0.0001$) times higher for mortalities over non-mortalities. Mortalities have significantly increased odds of having multiple complex chronic conditions. Table 5 differentiates the presence of concomitant CCC in patients with GIB as principal as opposed to secondary diagnosis whereas Table 2 summarizes the distribution of CCCs between mortality and non-mortality patients.

Mortalities had significantly higher odds of perforation as well (OR = 5.505, 95%CI: 1.717-17.650, $P = 0.021$). There was a significant association between mortality and diagnosis of sepsis during the encounter and mortality (OR = 2.583, 95%CI: 1.823-3.659, $P < 0.0001$). Mortalities had substantially higher odds of shock (OR = 3.585, 95%CI: 2.489-5.163, $P < 0.0001$), but this effect was modified by vasopressin. Mortalities had 4.834 times the odds of experiencing shock and receiving vasopressin compared to shock alone over non-mortalities (95%CI: 2.729-8.562, $P < 0.0001$). Vasopressin is primarily used to treat shock in critically ill children[3]. Higher mortality in patients receiving vasopressin and shock does not necessarily represent a causal chain - it may merely be highlighting the pattern that severe cases of shock were more frequently given vasopressin. The strength of the association is striking and the administration of vasopressin in shock cases did not associate with significantly improved outcomes. The effect of vasopressin on GI bleeds and shock warrants further investigation in pediatric patients.

Receiving octreotide or vasopressin was significantly

associated with having a portal hypertension diagnosis [χ^2 (1) = 3261.5, P < 0.0001], and with having varices with bleeding [χ^2 (1) = 2477.2, P < 0.0001]. Receiving octreotide within the first 24 h was associated with a 2.934 fold increase in odds of death (OR = 2.936, 95%CI: 1.981-4.351, P < 0.0001). This is more likely an indicator of severity of illness and early treatment as more severe patients who would eventually expire received more aggressive treatment.

Factors affecting mortality in GIB as principal, admitting or present on admit: As a sensitivity analysis, we isolated only those patients whose principal diagnosis was a GIB, or the GIB was present on admit or the admitting diagnosis to see if the trends observed associations pertained to a more refined group of GIB patients (n = 15539, 185 mortalities). After applying the model-fitting procedure, the final model yielded results similar to those observed for the full cohort of GIB patients in PHIS, with most of the associations being strengthened (the exception being CCCs), suggesting the secondary GIB may have biased estimates toward the null Age, race, and gender were not statistically significantly associated with mortality when controlling for other covariates (P = 0.999, P = 0.999, P = 0.937, respectively). Again, residing in an urban area, administration of H_2RA on day 0 or 1, chronic liver disease, and the interaction between endoscopy and chronic liver disease were not significant and did not improve model fit [χ^2 (5) = 4.527, P = 0.476]. Perforation-type injury and GIB present on admission were not significant and subsequently removed from the final model [χ^2 (2) = 3.644, P = 0.162].

In this smaller cohort, patients who died were even less likely to have undergone an endoscopic procedure (OR = 0.327, 95%CI: 0.202-0.539, P < 0.0001). Mortalities had marginally lower odds of receipt of a PPI on the first or second day of admission (OR = 0.613, 95%CI: 0.417, 0.902, P = 0.051). Receiving octreotide on the first or second day of admission was associated with an increase in odds of death (OR = 2.219, 95%CI: 1.286-3.831, P = 0.025).

The strength of the association with CCCs was not quite as pronounced in the smaller cohort. The odds of having 1 or 2 CCCs compared to 0 CCCs were 8.710 times higher for mortalities over non-mortalities (95%CI: 3.861-19.651, P < 0.0001), and the odds of having 3 or more CCCs compared to 0 CCCs was 24.098 (95%CI: 10.778-53.884, P < 0.0001) times higher for mortalities over non-mortalities. Mortalities have significantly increased odds of having multiple complex chronic conditions in the smaller cohort, but the associations are less strong.

There was a significant association between mortality and diagnosis of sepsis during the encounter (OR = 2.040, 95%CI: 1.197-3.477, P = 0.044). Mortalities had substantially higher odds of shock (OR = 5.426, 95%CI: 3.212-9.168, P < 0.0001) but this effect was modified by vasopressin, with a stronger association marked in the smaller cohort. Mortalities had 12.090 times the odds of experiencing shock and receiving vasopressin compared to shock alone over non-mortalities (95%CI: 5.327-27.442, P < 0.001).

Factors associating with endoscopy: In a separate model, we examined the association between various patient characteristics and whether or not the patient underwent endoscopy. A total of 5939 patients received endoscopy. Supplementary Table 2 stratifies therapeutic endoscopy type. The vast majority of endoscopic procedures were esophagogastroduodenoscopy. We adjusted the model for those factors relating to mortality and severity (shock, sepsis, packed red blood cell transfusion, GIB diagnosis present on admit, and GIB diagnosis as principal diagnosis). We found that those patients with chronic liver conditions were more likely to undergo endoscopy (OR = 2.378, 95%CI: 1.970-2.869, P < 0.0001), which may explain partially why this factor was not found significant in the mortality model. If endoscopy is protective and patients with chronic liver disease are more likely to undergo endoscopy, it stands to reason that they will then be less likely to die. We also found that living in a rural area was positively associated with endoscopy compared to living in an urban area (OR = 1.196, 95%CI: 1.076-1.329, P = 0.007). Compared to non-Hispanic white patients, Hispanics were 18.5% less likely to have undergone an endoscopic procedure (OR = 0.815, 95%CI: 0.737-0.900, P = 0.001). Age was also significantly associated with endoscopy, with a 6.39% increase in odds for every additional year (OR = 1.064, 95%CI: 1.058-1.070, P < 0.0001). Patients with 1 or 2 CCCs did not have increased odds of endoscopy (P = 0.999), but those with 3 or more had a 24.53% reduction in the odds of endoscopy (OR = 0.755, 95%CI: 0.686-0.830, P < 0.0001). Perforation injuries were also far less likely to undergo endoscopy (OR = 0.169, 95%CI: 0.116-0.247). GIBs as the principal diagnosis were associated with higher odds of endoscopy (OR = 2.626, 95%CI: 2.448-2.817, P < 0.0001). Those who underwent endoscopy were more likely to have experienced shock (OR = 1.752, 95%CI: 1.409-2.179, P < 0.0001), but less likely to have become septic (OR = 0.473, 95%CI: 0.370-0.604, P < 0.0001).

DISCUSSION

This is the first study describing the demographic and clinical characteristics of pediatric patients with GI hemorrhage in tertiary referral pediatric centers. In our cohort more than 75% of patients with GIB presented with hematemesis or blood in the stool. Mortality at, or before 3 d was more likely in patients with GIB as a primary diagnosis, and in this subgroup mortality was highest in the first 7 d of admission. The mortality in patients with a principal diagnosis of GIB was

0.37% whereas the mortality in patients with GIB as a secondary diagnosis was 2.96% signalling that GIB can be a terminal event in children with other severe disease processes.

We also found that in pediatric patients, race, gender, and age were not significantly associated with mortality. Death was most strongly associated with shock, sepsis, multiple complex chronic conditions and use of vasopressin and octreotide, although these pharmaceutical treatments may represent aggressive treatment of haemorrhage (octreotide) or hemodynamic support (vasopressin) for severely ill patients.

Our observations in children are analogous to published studies in adults showing mortality to be many times higher for upper GIB complicating the inpatient course in the presence of comorbidities[14]. In our cohort, we could not determine whether the increased mortality in patients with multiple chronic comorbidities was related to the GIB event or was intrinsic to the medical frailty of these patients. However, we observed the association to be consistent between both patients with any diagnosis of GIB as well as the more focused group with primary GIB diagnosis. GIB patients with multiple chronic illnesses are at incremental risk, and mortality in patients with primary GIB is significantly associated, albeit less robustly, with multiple complex chronic conditions than children with a secondary diagnosis. More specifically, our observations support the validity of the Sheffield Scoring System which identifies significant pre-existing condition as an independent determinant of the need for therapeutic endoscopy[11].

The observed increased mortality associated with GIB with chronic comorbidities and infection can be explained through several mechanisms. For example, the strong relationship between sepsis and mortality with GIB may relate to the development of disseminated intravascular coagulation that would exacerbate bleeding. Conversely septicemia may be a terminal complication in a child with multi-organ injury from bleeding-hypovolemic shock. Similarly oncologic comorbidities signal a greater degree of overall debility as a function of immunosuppression, impaired fluid and electrolyte balance, poor nutrition, suppressed erythropoiesis and several potential iatrogenic factors impacting homeostatic responses.

We could not, in this analysis, confirm a relationship between GIB related admission mortality and distance travelled to care as defined by rural compared with urban address; a significant relationship was noted when analysis was performed looking at all GIB associated mortality, this was not borne out when the cohort was defined by admitting or principal diagnosis.

Endoscopy was associated with lower mortality, as was the administration of a PPI on the first or second day of admission. Mortality was also lower for patients with GIB that was present on admission or the principal diagnosis, supporting the impression that GIB can be viewed as an ominous complication defining a generally more dismal outcome in children.

We did find that certain patients were more likely to undergo endoscopy. Endoscopy during admission with GIB diagnosis was significantly protective (OR = 0.49); the effect was most pronounced in children with a principal diagnosis of GIB (OR = 0.28). Chronic liver disease patients were much more likely to undergo endoscopy, which may explain why this comorbidity, in turn, was not found to be significantly associated with mortality although it is a known risk factor. Racial and urban *vs* rural differences were also noted. Hispanics were significantly less likely to undergo endoscopy during admission with GIB and mortality with GIB is lowest in non-Hispanic white children.

The sensitivity analysis yielded similar results when we focused on a smaller cohort of patients with principal diagnosis of GIB, admitting diagnosis of GIB, or GIB present on admit. The purpose of examining the smaller cohort was to exclude as much as possible those secondary GIB cases that arose as complication of a non-GIB disease course. We found even stronger association for most of the covariates, suggesting possible masking and bias toward the null by including more complex and severely ill patients whose hospital stay was not chiefly attributed to a GIB.

As a retrospective observational study using primarily administrative billing data, there are several limitations to the study. The use of ICD-9 diagnosis codes has been shown to be sensitive and specific for some conditions and procedures including gastrointestinal hemorrhage[15] but are unknown for all ICD-9 codes. Coding practices may also vary within hospitals, and the reliability of these codes depends on proper documentation. Substantial risk factors or severity factors may be missing from the data.

In summary, we have reported on the mortality associated with admission for acute GI hemorrhage in children. Gastrointestinal hemorrhage can be fatal but more often defines deterioration in a child with other, especially multiple, comorbidities. Intense pharmacologic support associates with mortality underscoring the escalation of therapy with increased clinical compromise. Endoscopy was consistently protective from mortality, and the timing, scope and therapeutic goals of endoscopy in GI hemorrhage are still to be precisely defined and universally applied in children. Emerging scoring systems and prospective implementation of such may go some way to identifying and stratifying the protective effects of endoscopy in children. This study offers new impetus to aggressive, including endoscopic, management.

COMMENTS

Background

The presentation, course and outcome of gastrointestinal haemorrhage in children compared with adults remains poorly characterized; little is known about factors related mortality associated with gastrointestinal bleeding in children and this impedes an evidence based approach to management in this population.

Research frontiers

Upper gastrointestinal haemorrhage, younger age at presentation and multiple comorbidities are associated with admission, whereas chronic illness, need for transfusion and large haemoglobin drop signal the need for endoscopy during admission. Mortality with gastrointestinal haemorrhage is most frequently associated with esophageal or intestinal perforation.

Innovations and breakthroughs

Gastrointestinal haemorrhage in children resulting in admission is associated with chronic comorbidities, most notably gastrointestinal, liver and cardiovascular disorders. Mortality is greater with more comorbid conditions at admission and more aggressive pharmacologic intervention whereas endoscopy during the admission was protective.

Applications

The prognosis for patients with gastrointestinal haemorrhage complicating the inpatient course especially in children with sepsis or multiple chronic comorbidities and requiring more aggressive hemodynamic support is especially guarded.

Peer-review

This manuscript is a very well designed and conducted retrospective study. There are outstanding information for clinical practice and important clues for prospective trials.

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ORIGINAL ARTICLE

Retrospective Study

Optimizing hepatitis C virus treatment through pharmacist interventions: Identification and management of drug-drug interactions

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Author contributions: Langness JA and Nguyen M contributed equally to this work; Wieland A, Everson GT and Kiser JJ contributed to the original project proposal and identification of key components; Kiser JJ is senior author and oversaw the initial proposal, design, statistical analysis and editing and formatting of manuscript; Langness JA and Nguyen M contributed to data collection, statistical analysis, and manuscript; Wieland A, Everson GT and Kiser JJ edited manuscript; all authors have read and approved final version to be published.

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Informed consent statement: As this was a retrospective review that involved de-identified data and did not affect patient care. Therefore informed consent was not required.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at jennifer.kiser@ucdenver.edu. All data associated with this study are de-identified and no personal health information is available through the dataset.

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Abstract

AIM

To quantify drug-drug-interactions (DDIs) encountered in patients prescribed hepatitis C virus (HCV) treatment, the interventions made, and the time spent in this process.

METHODS
As standard of care, a clinical pharmacist screened for DDIs in patients prescribed direct acting antiviral (DAA) HCV treatment between November 2013 and July 2015 at the University of Colorado Hepatology Clinic. HCV regimens prescribed included ledipasvir/sofosbuvir (LDV/SOF), paritaprevir/ritonavir/ombitasvir/dasabuvir (OBV/PTV/r + DSV), simeprevir/sofosbuvir (SIM/SOF), and sofosbuvir/ribavirin (SOF/RBV). This retrospective analysis reviewed the work completed by the clinical pharmacist in order to measure the aims identified for the study. The number and type of DDIs identified were summarized with descriptive statistics.

RESULTS
Six hundred and sixty four patients (83.4% Caucasian, 57% male, average 56.7 years old) were identified; 369 for LDV/SOF, 48 for OBV/PTV/r + DSV, 114 for SIM/SOF, and 133 for SOF/RBV. Fifty-one point five per cent of patients were cirrhotic. Overall, 5217 medications were reviewed (7.86 medications per patient) and 781 interactions identified (1.18 interactions per patient). The number of interactions were fewest for SOF/RBV (0.17 interactions per patient) and highest for OBV/PTV/r + DSV (2.48 interactions per patient). LDV/SOF and SIM/SOF had similar number of interactions (1.28 and 1.48 interactions per patient, respectively). Gastric acid modifiers and vitamin/herbal supplements commonly caused interactions with LDV/SOF. Hypertensive agents, analgesics, and psychiatric medications frequently caused interactions with OBV/PTV/r + DSV and SIM/SOF. To manage these interactions, the pharmacists most often recommended discontinuing the medication (28.9%), increasing monitoring for toxicities (24.1%), or separating administration times (18.2%). The pharmacist chart review for each patient usually took approximately 30 min, with additional time for more complex patients.

CONCLUSION
DDIs are common with HCV medications and management can require medication adjustments and increased monitoring. An interdisciplinary team including a clinical pharmacist can optimize patient care.

Key words: Clinical pharmacist; Drug-drug interaction; Hepatitis C virus treatment

Core tip: Identification and management of potential drug-drug interactions (DDI) is a critical aspect of current hepatitis C virus (HCV) treatment. This retrospective analysis of patients prescribed common HCV treatments identifies DDIs and the interventions made by the clinical pharmacist, as well as the approximate time required to complete these activities. This novel review illustrates that DDIs are common in this population. Identification and management of DDIs is resource intensive and requires medication adjustments and increased monitoring. An interdisciplinary care team including a clinical pharmacist is critical to optimize patient care for new HCV therapies.

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INTRODUCTION

Approximately 4.6 million Americans are estimated to have been exposed to the hepatitis C virus (HCV), and 3.5 million of those have active chronic infection[1]. The "baby boomer" generation (persons born between 1945 and 1965) have the highest incidence of chronic HCV infection *vs* any other age group[2]. Fifteen to twenty percent of people infected with chronic HCV progress to liver cirrhosis within twenty years, which may lead to end-stage liver disease or hepatocellular carcinoma[3]. In the United States, at least 35% of patients on the liver transplant wait list have chronic HCV[4]. HCV-associated mortality is close to 500000 deaths per year world-wide[5]. The previous standard of care, treatment with peginterferon and ribavirin, had significant challenges to treatment including serious adverse events, non-oral administration, and low efficacy rates. Direct-acting antivirals (DAAs) have improved the treatment landscape through increased efficacy, improved safety and tolerability, and all-oral administration. However, drug-drug interactions (DDIs) are a significant challenge and managing the interactions can be complex and time-consuming[6].

Pharmacists are recognized as important members of the health care team. Pharmacists' involvement in anticoagulation services, human immunodeficiency virus (HIV) care, cystic fibrosis, and diabetes has been shown to increase adherence, reduce pill burden and dosing frequency, and decrease medication-related errors[7-19]. The role of pharmacists in improving medical care and managing adverse effects in HCV treatment is well-recognized, but the impact of pharmacy interventions on therapeutic outcomes has not been adequately assessed in patients with HCV[20-24]. Furthermore, there is a lack of evidence for managing real-world DDIs for HCV treatments, especially with oral DAAs.

The University of Colorado Hospital Outpatient Hepatology Clinic has a clinical pharmacist imbedded within the interdisciplinary care team. The hepatologist first assesses a patient with chronic HCV infection, determining the stage of liver fibrosis, diagnosing advanced liver disease or cirrhosis, investigating any

disease complications, ordering all relevant baseline labs, and prescribing HCV treatment. The clinical pharmacist then reviews each patient prescribed HCV treatment to ensure correct dosing and dose adjustments as needed based on hepatic and renal function, to minimize therapeutic duplication, and to identify and manage DDIs. Each HCV medication has specific interactions with cytochrome P450 enzymes as well as transporters; and these medications can act as both victims and perpetrators in a number of DDIs. Potential DDIs are assessed using various resources including co-administration studies, medication package inserts, medication databases, and online tools such as www.hep-druginteractions.org. In situations where co-administration has not been studied, the pharmacology of each medication was reviewed to determine the potential for DDIs. Unfortunately, many herbal supplements lack data on pharmacokinetics and drug-drug interaction potential. If an herbal supplement did not have adequate data to ensure safe coadministration, the recommendation was often made to discontinue during HCV treatment. When managing DDIs, patient-specific factors such as vital signs, laboratory values, and concurrent use of other medications were accounted for. The clinical pharmacist coordinates with the internal medication access team to gain approval of the medication through the patient's prescription insurance plan or patient assistance programs. Once the patient is able to obtain medication, he or she meets with a physician assistant and the clinical pharmacist for a "medication start visit". During this visit, the specifics of treatment are reviewed, including the medication, dosing, administration, duration of treatment, potential side effects, and monitoring schedule. Clinically significant DDIs are reviewed with the patient and managed appropriately. The patient is then assessed through treatment by the physician assistant in conjunction with the clinical pharmacist.

Within the context of the role of the clinical pharmacist on the interdisciplinary team, the purpose of this study was to quantify (1) the type of DDI commonly encountered in patients prescribed HCV treatment in an academic outpatient hepatology clinic; (2) the interventions made; and (3) the time spent in identification and management of DDIs.

MATERIALS AND METHODS

This retrospective review identified all patients with chronic HCV infection who were prescribed HCV treatment at the University of Colorado Outpatient Hepatology Clinic between November 2013 and July 2015. Patients were included regardless of their HCV genotype and stage of liver fibrosis. DAAs prescribed during the time period included ledipasvir/sofosbuvir (LDV/SOF), paritaprevir/ritonavir/ombitasvir/dasabuvir (OBV/PTV/r + DSV), simeprevir/sofosbuvir (SIM/SOF), and sofosbuvir/ribavirin (SOF/RBV). Ribavirin may or may not have been used with the first three regimens. Patients were excluded from the study if they were coinfected with HIV or were post-liver transplant.

Demographic data including age, body mass index (BMI), and self-identified gender, race, and ethnicity were collected using the electronic medical record. HCV genotype and stage of liver fibrosis were recorded. Patients were classified as minimal to moderate fibrosis (Stage 0-2), advanced fibrosis (Stage 3), and cirrhosis (Stage 4) including both compensated and decompensated[25]. Baseline medications for each patient were collected, including prescription medication, over-the-counter medication, and vitamin and herbal supplements. Combination products such as multivitamins, vitamin-mineral supplements, and vitamin-mineral-herbal supplements were classified as one product. The number, type, and recommended management of DDIs were recorded for each patient. Baseline medications that were involved with DDIs were classified into nine categories. These categories include analgesics, hypertension/heart failure agents, anticonvulsants, psychiatric agents, proton pump inhibitors (PPIs)/H_2-receptor antagonists (H_2RA), antacids, steroids, vitamin and herbal supplements, and others. The number and type of interactions were summarized with descriptive statistics, due to the heterogeneous nature of the retrospective study.

Management of interactions was classified as: (1) discontinue medication; (2) increase monitoring; (3) alter administration time; (4) separate administration; (5) decrease dose; and (6) continue. The approximate time required for the identification, assessment, and management for clinical pharmacist review was recorded.

RESULTS

664 patients fit the inclusion and exclusion criteria: 369 with LDV/SOF, 48 with OBV/PTV/r + DSV, 114 with SIM/SOF, and 133 with SOF/RBV. Patients were 57% male and averaged 56.7 years old. 83.4% of patients identified as Caucasian, 6.44% as Black or African American, 1.89% as Asian, 0.30% American Indian or Alaska Native, and 7.97% as other or unavailable. 87% identified as Non-Hispanic and 13% Hispanic. The majority (51.5%) of the patients in the study were cirrhotic. See Table 1 for full demographic information.

Overall, 5217 medications were reviewed (7.86 medications per patient) and 781 interactions identified (1.18 interactions per patient) (Table 2). The average number of medications for each regimen was similar and ranged from 6.50 (OBV/PTV/r + DSV) to 8.79 (SIM/SOF). The average number of DDIs was lowest for SOF/RBV with 0.17, then 1.28 and 1.48 for LDV/SOF and SIM/SOF, respectively. OBV/PTV/r + DSV had the most DDIs per patient with 2.48. When accounting for stage of liver disease, the number of medications

Table 1 Baseline characteristics

Number of patients	664
Age (mean, yr)	56.7%
Gender	
Female	42.8%
Male	57.2%
Race	
Caucasian	83.4%
African American or Black	6.44%
Asian American	1.89%
American Indian or Alaska Native	0.30%
Other and unavailable	7.97%
Ethnicity	
Hispanic	13.6%
Non-Hispanic	86.4%
Number of patients on DAAs	
LDV/SOF	369%
OBV/PTV/r + DSV	48%
SIM/SOF	114%
SOF/RBV	133%
Fibrosis stage	
≤ Stage 2 (minimal to moderate fibrosis)	35.8%
Stage 3 (advanced fibrosis)	10.8%
Stage 4 (cirrhosis)	51.5%
Unknown or unavailable	1.90%

LDV: Ledipasvir; SOF: Sofosbuvir; OBV: Ombitasvir; PTV: Paritaprevir; DSV: Dasabuvir; SIM: Simeprevir; RBV: Ribavirin; DAA: Direct acting antiviral.

per patient trended upward from 6.50 for patients with minimal fibrosis to 8.99 for patients with cirrhosis (Table 3). Despite the greater number of concomitant medications, there was a similar average number of DDIs in those with minimal *vs* more advanced disease. The most common interactions (identified as ≥ 10%) were vitamin and herbal supplements (284/781, 36.4%), PPI/H_2RA agents (117/781, 15.0%), and other products (126/781, 16.1%). Table 4 summarizes the interactions amongst the different drug classes. Figure 1 shows the recommendations made for the management of DDIs.

LDV/SOF

In 369 patients prescribed LDV/SOF, 472 drug-drug interactions were identified. Common interactions (defined as ≥ 10%) with LDV/SOF included antacids (72/472, 15.3%), PPI/H_2RA agents (107/472, 22.7%), and vitamin/herbal supplements (227/472, 48.1%). Ledipasvir, an NS5A inhibitor, is better absorbed in an acidic environment. When omeprazole 20 mg was administered once daily 2 h prior to LDV, area underneath the curve (AUC) decreases to 0.58[26]. Therefore, absorption is decreased with any medications that affect stomach acidity. Overall, interactions with antacids and PPI/H_2RA agents occurred with (118/472, 25.0%) of our patients prescribed LDV/SOF. This interaction can be challenging for multiple reasons. These medications are available without prescription and often patients can forget to report them during medication reconciliation. Each patient prescribed LDV/SOF was explicitly asked if they were taking any prescription or non-prescription medications for heartburn or gastric esophagitis reflux diseas, or any other type of antacids and PPI/H_2RA agents. Another challenging aspect is that PPIs are recommended for patients post banding ligation, a comorbidity common in patients with advanced liver disease. In order to manage the DDIs with PPI/H_2RA agents, 54.2% were on the appropriate dose but were required to alter administration time; 40.2% were required to both decrease dose and alter administration time (40.2%). Supplements such as milk thistle, cod liver, krill oil, garlic cap, turmeric, and saw palmetto were recommended to be put on hold (70.4%) or separated from LDV/SOF (28.3%) administration time. Although there were few interactions with anticonvulsants (5/472, 0.85%), each occurrence was associated with a contraindication with LDV/SOF. For patients taking carbamazepine, oxcarbazepine, phenobarbital, and phenytoin, the recommendation was made to transition to an alternative anticonvulsant prior to initiation of HCV treatment.

OBV/PTV/r + DSV

Analgesics (22/119, 18.5%), vitamins and herbal supplements (21/119, 17.6%), and hypertensive agents (19/119, 16.0%) frequently interact with OBV/PTV/r + DSV. Managing interactions with analgesics such as morphine, oxycodone, tramadol, and hydrocodone, can be complicated due to the variability in dosing, patient response, and opioid tolerance. In order to manage the DDIs with analgesics, the dose was most often reduced and monitoring increased (81.8%). Supplements were recommended to be discontinued during HCV therapy (57.1%), separated by at least four hours (28.6%), or increased monitoring for adverse events (14.3%). Hypertensive agents including furosemide and amlodipine can have a greater affect due to the DDI with OBV/PTV/r + DSV. Depending on the dose of the hypertensive medications and the patient's blood pressure, the medications were continued at the same dose with increased monitoring for hypotension (7/19, 36.8%) or to decrease the dose (12/19, 63.2%) in anticipation for increased plasma concentration levels of the hypertensive agents. Other medication classes that interacted were erectile dysfunction agents (tadalafil), lipid lowering agents (pravastatin, rosuvastatin), allergy symptom medications (cetirizine), and insomnia agents (trazodone). Depending on the medication and indication, often the recommendation was to decrease the dose (23.5%) or discontinue the agent (11.8%).

SIM/SOF

Analgesics (21.3%), hypertensive agents (13.0%), psychiatrics (20.1%), and vitamins and herbal supple-

Table 2 Drug-drug interactions identified from baseline medication list

Regimen	*n* = 664	Total number of meds	Total number of interactions, *n* (%)	Average number of meds per patient	Average number of interactions per patient	Contra-indications
LDV/SOF	369	2996	472 (15.8)	8.12	1.28	7
OBV/PTV/r + DSV	48	312	119 (38.1)	6.50	2.48	4
SIM/SOF	114	1002	169 (16.8)	8.79	1.48	19
SOF/RBV	133	964	21 (2.2)	7.25	0.16	1

LDV: Ledipasvir; SOF: Sofosbuvir; OBV: Ombitasvir; PTV: Paritaprevir; DSV: Dasabuvir; SIM: Simeprevir; RBV: Ribavirin.

Table 3 Drug-drug interactions identified per fibrosis stage

Fibrosis stage	*n* = 664	Total number of medications	Total number of interactions *n* (%)	Average number of medications per patient	Average number of interactions per patient
Minimal fibrosis (Stage 0-2)	232	1508	249 (16.5)	6.50	1.07
Advanced fibrosis (Stage 3)	72	575	91 (15.8)	7.99	1.26
Cirrhosis (Stage 4)	341	3066	425 (13.8)	8.99	1.25

Table 4 Drug classes of medications identified as drug-drug interactions from baseline medication list *n* (%)

Drug class	Affected portion of the cohort *n* = 664
PPI/H2RA agents	117 (17.6)
Antacids	72 (10.8)
Vitamin and herbal supplements	284 (42.7)
Hypertensive agents	53 (8.0)
Analgesics	67 (10.1)
Psychiatric agents	46 (6.9)
Anticonvulsants	4 (0.6)
Steroids	12 (1.8)
Others	126 (19.0)

PPI: Proton pump inhibitor; H2RA: H2-receptor antagonists.

ments (10.65%) frequently interact with SIM/SOF. Even though most of these medications have not been evaluated for DDIs with SIM/SOF, we anticipate the plasma concentration of these medications to increase due to mild inhibition of CYP3A4 and OAT1B1 from simeprevir[27]. As a result, increase monitoring for side effects and decrease dose were frequently recommended for analgesics (76.3%; 21.1%), hypertensive agents (70.0%; 25.0%), and psychiatrics (82.4%; 14.7%). Herbal supplements such as St. John's wort and milk thistle are contraindicated with SIM/SOF. When administered with inducers or inhibitors of CYP3A4 enzyme, the plasma concentration of SIM is expected to change[28,29]. Therefore, both St. John's wort and milk thistle were recommended to be discontinued during the course of HCV therapy (100%). Other medications that had potential interaction with SIM/SOF were lipid lowering agents, anti-nausea medications, bladder dysfunction medications, antibacterial agents, and insomnia medications. Based on the metabolism of these medications, the most common recommendations were to increase monitoring for side effects (56.0%) or to reduce the dose (28.0%) of these medications (Figure 1).

SOF/RBV

SOF/RBV had the fewest identified DDIs of any regimen. Ribavirin has the fewest direct interactions for any of the HCV medications, and sofosbuvir has relatively few as well. Vitamins and herbal supplements that have not been studied with SOF/RBV represented the largest group of potential DDIs (81.8%). Since DDIs have not been evaluated with supplements such as milk thistle, turmeric, mushroom extract, and horny goat weed, it was recommended to discontinue these while on therapy for treating HCV infection (100%). Due to the DDI between sofosbuvir and carbamazepine, the contraindicated medication was discontinued prior to initiating HCV treatment in one patient.

Time management

The clinical pharmacist was able to record his time spent reviewing medications in 105 consults. These consults included LDV/SOF, OBV/PTV/r + DSV, and SOF/RBV, but did not include SIM/SOF. The time requirement increased with the complexity of the patient and the number of baseline medications and drug-drug interactions identified. Consults for patients prescribed OBV/PTV/r + DSV took the longest for the pharmacist to complete, averaging 30 min per consult. SOF/RBV and LDV/SOF were slightly shorter averaging 20 min per consult. This is consistent in relation to our data that shows more DDIs were identified in patients prescribed OBV/PRV/r + DSV.

DISCUSSION

Drug-drug interactions continue to be a considerable challenge for managing patients with HCV treatment. This study assessed the frequency and pharmacologi-

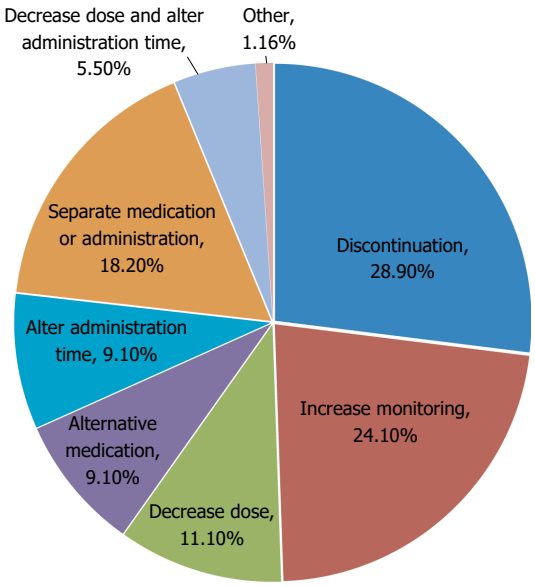

Figure 1 Recommendations for management of drug-drug interactions (*n* = 664). Other recommendations: continue, alternative medication and alter administration, decrease dose and increase monitoring, decrease dose and separate administration, and increase dose.

cal category of identified DDIs in real-world patients with HCV in addition to describing the management of the most common DDIs encountered with the DAAs. Published data on this topic are lacking. In 2010, a US insurance cohort showed the top four medication classes in patients with chronic HCV were analgesics and/or antipyretics, antidepressants, antivirals, and gastrointestinal agents including proton pump inhibitors[29]. Additionally, the study identified the average number of baseline medications as 9.0 and 11.4 in HCV treated and untreated patients, respectively. Höner Zu Siederdissen *et al*[30] published an account of potential drug-drug interactions in a cohort of patients in Hanover, Germany. Our result for the number of medications per patient at baseline study (7.86 medications per patient) was comparable with the US cohort. A significant number of DDIs predicted in our patient cohort were with analgesics (9%), antihypertensives (7%), and psychiatrics (6%). With the exception of psychiatrics and analgesics, this is comparable with both the US and German cohorts.

Use of over-the-counter medications and herbal supplements presents a challenge for assessing and managing DDIs in patients. Herbal products are gaining popularity in the US and there is a perception that herbal medications are safer than conventional medications[31]. These supplements can be recommended by people other than the patient's primary healthcare provider. Other times, patients will forget or not realize to inform the provider about non-prescription medications. In the HALT-C study, 44% of 1145 participants in the study with HCV infection admitted past or current use of herbal supplements[29]. Because the supplements are not prescribed, often they are not documented in the electronic medical records. Additionally, non-prescription medications and herbal supplements are rarely studied for interactions. When data were available or the potential interaction was minimal, the clinical pharmacist usually recommended continuing the product. However, if there were no data to support the safe use of the supplement with HCV treatment, the clinical pharmacist generally recommended it to be discontinued. This was especially true with supplements that had no clear benefit to the patient. Supplements such as multivitamins, fish oil, and probiotics that have shown benefits in certain populations, were most often recommended to separate from the DAAs in order to avoid any potential absorption interaction.

As with most hepatology clinics, the University of Colorado Hospital Hepatology Clinic largely acts as a consult service for liver disease management. As a result, providers through other clinics prescribe most concomitant medications. Adjusting or switching a medication due to DDIs can be relatively straightforward with certain medication classes such as lipid lowering agents and antihypertensive medications. However, management can be complex with other DDIs. All currently available HCV therapies have DDIs with certain anticonvulsants. These anticonvulsants are normally prescribed and managed through a specialty neurology clinic, sometimes outside of the health-system. The clinical pharmacist may not have access to the neurology clinic notes, and *vice versa*. Depending on the disease state being treated and specific patient factors, an alternative anticonvulsant that does not interact may not be appropriate. If an appropriate alternative is found, the switch can involve specific titration schedules and overlap, requiring management and specific monitoring. DDIs involving antipsychotic medication or other mental health medications are also complex. Patient responses are varied and sometimes unpredictable to certain agents, and exacerbation of mental health disease is a significant health concern. In these situations, it may be pertinent to choose a different HCV medication regimen with fewer interactions as opposed to switching the concomitant medications. Prescription insurance companies often have a specific formulary agent, and obtaining approval for an alternative HCV regimen can be challenging and time consuming.

Serious adverse events have been reported due to DDIs. This includes a case of rhabdomyolysis associated with telaprevir and simvastatin, renal failure related to the increased levels of tacrolimus after starting protease inhibitor therapy, new-onset diabetes due to the interactions between LDV/SOF and tenofovir, and severe bradyarrhythmias due to the interaction between amiodarone and sofosbuvir[32-35]. Although relatively rare, these interactions can lead to

very serious patient harm or potentially death. These cases illustrate the importance of having knowledge of possible drug-drug interactions to prevent severe side effects. In our study, there were no documented significant adverse events due to drug-drug interactions. By understanding the pharmacodynamics and pharmacokinetic profiles of the drugs involved with interactions, therapy can be safely and effectively managed in patients with HCV infection.

This study is limited by the retrospective nature of the review. Additionally, it is a single-center study at an academic medical center and regional transplant center, thereby limiting the relatability of the results to smaller clinics with less complexity. Additionally, the region lacks significant diversity and the result may differ with patients of different racial and ethnic backgrounds. Potential selection bias can also limit the results, as the patients were not randomized to each medication regimen but selected based on patient specific characteristics, medication regimen characteristics, provider experience and judgment, and insurance formulary. For patients with cirrhosis, depending on compensation and Child-Pugh Score, he or she may not be eligible to receive certain medication regimens. Due to all of the reasons, only descriptive statistics were used to analyze the results. Additionally, measuring the time requirement for the pharmacists to complete a consult was challenging. Anecdotally, as the pharmacist was more familiar with the medications and common DDIs, the time spent per patient was shortened. Additionally, due to the nature of a consult position, interruptions were common making it hard to capture the true amount of time spent per consult. For these reasons, caution should be warranted when applying these data to other clinics.

In conclusion, DDIs are common in patients prescribed HCV medications and the involvement of a clinical pharmacist can be beneficial to the interdisciplinary hepatology team. Identification and management of DDIs is resource intensive and requires medication adjustments and increased monitoring. Clinical pharmacists can encourage preventive measures on reducing HCV transmission, increase education adherence, assist in initiating HCV treatment, assist in monitoring clinical and adverse effects, and facilitate medication acquisition.

COMMENTS

Background

Identification and management of potential drug-drug interactions (DDIs) is a critical aspect of current hepatitis C virus (HCV) treatment. Direct-acting antivirals (DAAs) have improved the treatment landscape through increased efficacy, improved safety and tolerability, and all-oral administration. However, DDIs are a significant challenge and managing the interactions can be complex and time-consuming.

Innovations and breakthroughs

Drug-drug interactions continue to be a considerable challenge for managing patients with HCV treatment. This study assessed the frequency and pharmacological category of identified DDIs in real-world patients with HCV in addition to describing the management of the most common DDIs encountered with the DAAs.

Applications

This retrospective analysis of patients prescribed common HCV treatments identifies DDIs and the interventions made by the clinical pharmacist, as well as the approximate time required to complete these activities. This novel review illustrates that DDIs are common in this population. Identification and management of DDIs is resource intensive and requires medication adjustments and increased monitoring. An interdisciplinary care team including a clinical pharmacist is critical to optimize patient care for new HCV therapies.

Peer-review

Drug-drug interactions continue to be a considerable challenge for managing patients with HCV treatment. In this study the authors assessed the frequency and pharmacological category of identified drug-drug-interactions in real-world patients with HCV. This study suggests an interdisciplinary approach for managing DDIs. In my opinion, publication will be valuable.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy and safety of limited endoscopic sphincterotomy before self-expandable metal stent insertion for malignant biliary obstruction

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Abstract

AIM

To evaluate the safety and efficacy of limited endoscopic sphincterotomy (ES) before placement of self-expandable metal stent (SEMS).

METHODS

This was a retrospective analysis of 244 consecutive patients with unresectable malignant biliary obstruction, who underwent placement of SEMSs following limited ES from December 2008 to February 2015. The diagnosis of malignant biliary obstruction and assessment of patient eligibility for the study was established by a combination of clinical findings, laboratory investigations, imaging and pathological results. All patients were monitored in the hospital for at least 24 h following endoscopic retrograde cholangio pancreatography (ERCP). The incidence of immediate or early post-ERCP complications such as post-ERCP pancreatitis (PEP) and bleeding related to limited ES were considered as primary outcomes. Also, characteristics and complications according to the cancer type were classified.

RESULTS

Among the 244 patients included, the underlying diagnosis was cholangiocarcinoma in 118 patients,

pancreatic cancer in 79, and non-pancreatic or non-biliary malignancies in the remaining 47 patients. Early post-ERCP complications occurred in 9 patients (3.7%), with PEP in 7 patients (2.9%; mild, 6; moderate, 1) and mild bleeding in 2 patients (0.8%). There was no significant association between the incidence of post-ERCP complications and the type of malignancy (cholangiocarcinoma *vs* pancreatic cancer *vs* others, P = 0.696) or the type of SEMS used (uncovered *vs* covered, P = 1.000). Patients who had more than one SEMS placed at the first instance were at a significantly higher risk of post-ERCP complications (one SEMS *vs* two SEMS, P = 0.031). No other factors were predictive of post-ERCP complications.

CONCLUSION

Limited ES is feasible and safe, and effectively facilitates the placement of SEMS, without any significant risk of PEP or severe bleeding.

Key words: Endoscopic sphincterotomy; Endoscopic retrograde cholangio pancreatography; Complications; Pancreatitis; Bleeding; Cholestasis; Self-expandable metal stent

Core tip: The role of routine endoscopic sphincterotomy (ES) is still controversial in biliary stenting and there is a lack of systematic study for the extent of ES and its correlation with the incidence of complications. We retrospectively evaluated the safety and efficacy of limited ES before self-expandable metal stent insertion. We have proved in this study that limited ES doesn't increase the risk of post-procedure complications such as post-endoscopic retrograde cholangio pancreatography pancreatitis and bleeding. Also, it is advantageous in facilitating the more complex stenting procedures. Therefore, limited ES can be a safe, feasible, and effective therapeutic strategy in the placement of self-expandable metal stent.

Nam HS, Kang DH, Kim HW, Choi CW, Park SB, Kim SJ, Ryu DG. Efficacy and safety of limited endoscopic sphincterotomy before self-expandable metal stent insertion for malignant biliary obstruction. *World J Gastroenterol* 2017; 23(9): 1627-1636 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1627.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1627

INTRODUCTION

Malignant biliary obstruction is mainly caused by cholangiocarcinoma, pancreatic cancer, gallbladder cancer, and metastatic disease. The prognosis is very poor because the lesions are unresectable at diagnosis in the majority of these patients, with less than 20% of the patients being suitable for surgical resection[1]. Endoscopic retrograde cholangiopancreatography (ERCP) along with biliary stenting is a minimally invasive procedure for palliation of biliary obstruction that provides relief from jaundice and improves the quality of life of patients with unresectable malignant biliary obstruction[2].

Self-expandable metal stents (SEMS), compared to plastic stents, have superior patency and are cost-effective options in selected preoperative patients or in patients whose life expectancy exceeds six months[3-5]. They are, however, reported to be associated with a higher incidence of pancreatitis[6,7]. Previous studies indicate that performing endoscopic sphincterotomy (ES) before stent insertion may lower the incidence of post-ERCP pancreatitis (PEP)[8-10]. ES may also facilitate cannulation of the bile duct during difficult ERCPs, reduce resistance to the passage of stents, improve immediate stent deployment, and increase the luminal diameter of the distal common bile duct (CBD)[9-12]. Many endoscopists routinely perform ES before SEMS placement. However, the role of routine ES before stenting is still controversial and no clear guidelines exist to govern its use. Additionally, ES is also an independent risk factor for complications such as pancreatitis, bleeding, and perforation, with a reported complication rate of approximately 10% and an overall direct or indirect procedure-related mortality of 0.42%, even when performed by experienced endoscopists[2,13-17]. However, an accurate assessment of the incidence of complications based on the extent of ES is difficult to make owing to the lack of such data in previous studies. Herein, we studied the incidence of early post-ERCP complications, such as PEP and bleeding following limited ES accompanying SEMS placement for biliary drainage in patients with malignant biliary obstruction.

MATERIALS AND METHODS

Patients

This was a retrospective analysis of all patients who underwent endoscopic biliary SEMS placement for the first time for malignant biliary obstruction at the Pusan National University Yangsan Hospital during the six-year period from December 2008 to February 2015. Patients that underwent transpapillary SEMS placement after limited ES for a diagnosis of distal or hilar malignant biliary strictures were included in this study. Diagnosis of the disease and assessment of patient eligibility for the procedure was based on a combination of clinical findings, laboratory investigations and radiological studies including computed tomography (CT) scan, magnetic resonance imaging (MRI) and/or endoscopic ultrasound (EUS). In case of without cholangitis, painless jaundice and/or pruritus, sometimes anorexia, weight loss and malaise were main clinical symptoms. The main laboratory parameters recorded were complete blood count (CBC), total bilirubin, liver

function tests including alanine aminotransferase, alkaline phosphatase, γ-glutamyltransferase and tumor markers such as CEA and CA 19-9. CT scan was performed for all patients as an initial test, while MRI was performed in all patients with suspicious malignant biliary strictures. MRI was not performed in uncooperative patients or if contraindicated owing to the presence of intracorporeal metallic device or foreign body. EUS was not routinely performed, and was limited to investigating indeterminate biliary strictures, nonvisible masses, or when tissue acquisition was required for definite diagnosis. Pathology results were reviewed in cases where biopsy was performed during ERCP or EUS. Exclusion criteria were previous ES or stent placement, coagulopathy (international normalized ratio > 1.5), low platelet count (< 50000/mL), current use of anticoagulant or antiplatelet drugs, severe cholangitis with or without septic shock, Billroth II anatomy or Roux-en-Y gastrojejunostomy, and severe heart or pulmonary disease. The study protocol was approved by the ethics committee of the Institutional Review Board of Pusan National University Yangsan Hospital (IRB No. 05-2015-081).

Study protocol

Patient characteristics including age, sex, history of previous procedures and baseline biochemical and hematological values were collected prior to performing ERCP. SEMS placement was performed in all patients by one of two experienced endoscopists (endoscopist A had performed > 10000 ERCPs over 20 years; endoscopist B had performed > 2000 ERCPs over 10 years). All patients received intravenous (IV) broad-spectrum antibiotics. Nafamostat mesilate (20 mg) was administered for all patients for preventing post-ERCP pancreatitis. Nonsteroidal anti-inflammatory drugs were not used routinely. All procedures were performed under conscious sedation by using IV midazolam and pethidine, with the patient in the supine or left lateral decubitus position. Cimetropium bromide 10 mg IV was administered to reduce duodenal peristalsis. All ERCPs were performed by using a standard side-viewing duodenoscope (JF-260 V or TJF-240; Olympus Optical Co., Ltd., Tokyo, Japan). Selective cannulation of the bile duct was achieved by using a pull-type double-lumen sphincterotome (Ultratome XL, Boston Scientific, Natick, Mass) or by a conventional ERCP catheter (Fluoro Tip, Boston Scientific), with or without a hydrophilic guidewire (0.025- or 0.035-inch Jagwire, Boston Scientific). A wire-guided cannulation technique was attempted first, followed by the conventional contrast-assisted cannulation technique if biliary cannulation was not achieved within 10 min. After successful guidewire placement, limited sphincterotomy was performed with blended current. Limited ES was defined as ES limited to one-third the extent of major ES. A metal stent was then inserted over the guidewire under fluoroscopic control. Stent length (4 cm to 12 cm) and the need for unilateral or bilateral stent placement were determined based on the location and length of the biliary stricture. Stent placement ensured that the stent spanned the stricture with either end of the stent extending a minimum of 1 cm beyond the stricture. In the case of distal biliary strictures, the distal end of SEMS was placed across the papilla with 1 cm of the distal end of the stent exposed in the duodenum. In the case of hilar biliary strictures, SEMS was placed above the sphincter of Oddi. All patients were monitored in the hospital for at least 24 h after ERCP to identify early post-ERCP complications. CBC, serum amylase, and lipase levels were routinely evaluated at 4 h and 24 h after the procedure. Endoscopy was performed to evaluate ES-related-bleeding on the day following stent placement. All adverse events were recorded. During the follow up period, ERCP was repeated on suspecting stent complications such as occlusion or migration.

Definitions

According to updated Tokyo guidelines (TG13) for diagnosis and severity grading of acute cholangitis, cholangitis was defined as fever and/or shaking chills, increased inflammatory response (abnormal white blood cell counts, increased serum C-reactive protein levels) and jaundice (total bilirubin ⩾ 2 mg/dL) or abnormal liver function tests (> 1.5 × upper limit of normal value)[18]. Severe cholangitis is defined as the presence of accompanying organ dysfunction caused by biliary sepsis, and requiring intensive care such as respiratory and circulatory support[18]. Limited ES was defined as sphincterotomy less than one-third the extent of major ES[19]. Definitions of individual post-procedure complications were according to the descriptions given by Cotton *et al*[20]. PEP was defined as new-onset or worsening abdominal pain lasting more than 24 h after the procedure, in conjunction with pancreatic enzyme (amylase and/or lipase) elevation that was at least three times the upper limit of the normal, with or without radiographic evidence of acute pancreatitis. The severity of PEP was graded by using the number of hospitalization days: mild, when hospitalization was prolonged by 2 to 3 d, moderate, by 4 to 10 d, and severe, by more than 10 d[20]. Bleeding was defined as the presence of melena or hematemesis, irrespective of the need for blood transfusion or repeat endoscopy. Mild bleeding was defined as hemoglobin drop within 2 g/dL, with no necessity for blood transfusion. The presence of bleeding was identified based on patient's history (melena or hematemesis) and a drop in hemoglobin level following the procedure. Perforation was considered as perforation of retroperitoneum or bowel walls documented by any of the radiographic techniques[21]. Complications were graded according to the grading system described by Cotton *et al*[20]. Early complications or adverse events were defined as any ERCP-related complications occurring within 30 d

Table 1 Patient characteristics and endoscopic retrograde cholangio pancreatography related data *n* (%) (*n* = 244)

Characteristics	Value
Age (yr), mean ± SD (range)	70.8 ± 10.2 (44-95)
Sex	
Male	130 (53.3)
Female	114 (46.7)
Aspirin	3 (1.2)
Total bilirubin (mg/dL), pre-procedure, mean ± SD (range)	7.09 ± 6.45 (0.2-28.9)
Normal	53 (21.7)
Elevated	191 (78.3)
Hyperamylasemia, pre-procedure	14 (5.7)
Cholangitis, pre-procedure	68 (27.9)
Diagnosis	
Cholangiocarcinoma	118 (48.4)
Hilar	75 (63.6)
Distal	43 (36.4)
Pancreatic cancer	79 (32.4)
Head	68 (86.1)
Body/Tail	11 (13.9)
Gallbladder cancer	21 (8.6)
Ampullary cancer	18 (7.4)
Hepatocellular carcinoma	3 (1.2)
Others	5 (2.0)
Pancreatic duct invasion	
Yes	85 (34.8)
No	159 (65.2)
Lymph node metastasis	
Yes	170 (69.7)
No	73 (29.9)
Pancreatic duct injection	
0	187 (76.6)
1-2	24 (9.8)
⩾ 3	33 (13.5)
ERPD	4 (1.6)
Stent success rate	244 (100)
Number of initially inserted SEMS	
1	230 (94.3)
2	14 (5.7)
Stent type	
Uncovered	190 (77.9)
Covered	54 (22.1)
Post-ERCP complication	
Present	9 (3.7)
Absent'	234 (95.9)
Post-ERCP complication type	
Pancreatitis	7 (2.9)
Mild / moderate	6 (2.5)/1 (0.4)
Bleeding, mild	2 (0.8)
Perforation	0 (0)
Post-ERCP hyperamylasemia	30 (12.3)
Stent complication	
None	199 (81.6)
Stent occlusion	44 (18.0)
Stent migration	1 (0.4)
Patency	
No further procedure	199 (81.6)
ERBD restent	1 (0.4)
SEMS restent	32 (13.1)
PTBD	12 (4.9)

SEMS: Self-expandable metal stent; ERPD: *Endoscopic retrograde pancreatic drainage*; ERBD: Endoscopic retrograde biliary drainage; PTBD: Percutaneous transhepatic biliary drainage.

of the procedure. Patency interval was defined as the period between the first SEMS deployment and the occurrence of stent complications such as occlusion or migration.

Statistical analysis

The primary outcomes measured were immediate or early complications within 30 d of the procedure. For inter-group differences, Student's *t*-test was performed for continuous variables, and χ^2 test or Fisher's exact test were performed for categorical variables. Results were considered statistically significant at a P value < 0.05. Data were analyzed by using SPSS software version 18.0 (SPSS, Chicago, IL, United States).

RESULTS

A total of 244 patients that underwent limited ES and biliary stenting for malignant biliary obstruction between December 2008 and February 2015 were included in the study. The etiology of malignant biliary obstruction included cholangiocarcinoma (n = 118, 48.4%), pancreatic cancer (n = 79, 32.4%), and others including gallbladder cancer (n = 21, 8.6%), ampullary cancer (n = 18, 7.4%), and hepatocellular carcinoma and metastatic cancer (n = 8, 3.2%). Mean age was 70.8 ± 10.2 (range, 44-95) years and 130 (53.3%) were males and 114 (46.7%) were females. Stents were successfully deployed in all patients.

Early post-ERCP complications occurred in 9 patients (3.7%), including PEP in 7 patients (2.9%; mild, 6; moderate, 1), and mild bleeding in 2 patients (0.8%). All patient with post-ERCP complications responded to conservative management. Stent occlusion and migration developed in 44 patients (18.0%) and 1 patient (0.4%), respectively. Patients with late complications underwent repeat ERCP or percutaneous transhepatic biliary drainage. Patient characteristics and ERCP related data are summarized in Table 1.

On categorizing patients into three groups on the basis of cancer location, PEP developed in 4 patients (3.4%, 4/118) with cholangiocarcinoma, 1 patient (1.3%, 1/79) with pancreatic cancer, and 2 patients (4.3%, 2/47) with non-pancreatic, non-biliary cancers (P = 0.681). There were no significant differences among these three groups as to the incidence of immediate or early complications (P = 0.696) (Table 2). In the cholangiocarcinoma group, the incidence of PEP was 4.0% and 2.3% with hilar and distal cholangiocarcinoma, respectively (P = 0.537). One patient with hilar cholangiocarcinoma had mild bleeding (Table 3). In the pancreatic cancer group, one patient had PEP and another had mild bleeding. Both complications developed in patients with pancreatic head cancer and none were reported in cases of pancreatic body and/or tail cancer (Table 4).

Table 2 Characteristics and complications according to the cancer type *n* (%)

	Cholangiocarcinoma *n* = 118	Pancreatic cancer *n* = 79	non-pancreaticobiliary cancer *n* = 47	*P* value
Age (yr), mean ± SD	73.5 ± 9.4	67.8 ± 10.4	69.3 ± 10.3	0.002
Hyperamylasemia, pre-procedure	6 (5.1)	3 (3.8)	5 (10.6)	0.273
Cholangitis, pre-procedure	28 (23.7)	19 (24.1)	21 (44.7)	0.021
Pancreatic duct invasion				< 0.001
Yes	12 (10.2)	64 (81.0)	9 (19.1)	
No	106 (89.8)	15 (19.0)	38 (80.9)	
Lymph node metastasis				0.345
Yes	77 (65.3)	58 (73.4)	35 (74.5)	
No	41 (34.7)	21 (26.6)	12 (25.5)	
Pancreatic duct injection				0.606
0	92 (78.0)	59 (74.7)	36 (76.6)	
1-2	8 (6.8)	9 (11.4)	7 (14.9)	
⩾ 3	18 (15.3)	11 (13.9)	4 (8.5)	
Number of initially inserted SEMS				0.004
1	106 (89.8)	79 (100.0)	45 (95.7)	
2	12 (10.2)	0 (0.0)	2 (4.3)	
Post-ERCP complication				0.696
Present	5 (4.2)	2 (2.5)	2 (4.3)	
Absent	113 (95.8)	77 (97.5)	45 (95.7)	
Post-ERCP complication type				0.914
Pancreatitis	4 (3.4)	1 (1.3)	2 (4.3)	0.681
Mild/moderate	3 (2.5)/1 (0.8)	1 (1.3)/0 (0)	2 (4.3)/0 (0)	
Bleeding, mild	1 (0.8)	1 (1.3)	0 (0)	
Perforation	0 (0)	0 (0)	0 (0)	
Post-ERCP hyperamylasemia	13 (11.0)	9 (11.4)	8 (17.0)	
Stent complication				0.539
None	90 (76.3)	70 (88.6)	39 (83.0)	
Stent occlusion	27 (22.9)	9 (11.4)	8 (17.0)	
Stent migration	1 (0.8)	0 (0)	0 (0)	
Patency				0.161
No further procedure	90 (76.3)	70 (88.6)	39 (83)	
ERBD restent	1 (0.8)	0 (0)	0 (0)	
SEMS restent	20 (16.9)	9 (11.4)	3 (6.4)	
PTBD	7 (5.9)	0 (0)	5 (10.6)	

SEMS: Self-expandable metal stent; ERBD: Endoscopic retrograde biliary drainage; PTBD: Percutaneous transhepatic biliary drainage.

Table 3 Rates of complications on biliary stenting with limited endoscopic sphincterotomy according to location of cholangiocarcinoma *n* (%)

	Hilar *n* = 75	Distal *n* = 43	*P* value
Post-ERCP complication type			0.717
Pancreatitis	3 (4.0)	1 (2.3)	
Mild/moderate	2 (2.7)/1 (1.3)	1 (2.3)/0 (0)	
Bleeding, mild	1 (1.3)	0 (0.0)	
Perforation	0 (0.0)	0 (0.0)	
Post-ERCP hyperamylasemia	7 (9.3)	5 (11.6)	
Stent complication			0.756
None	57 (76.0)	33 (76.7)	1.000
Stent occlusion	17 (22.7)	10 (23.3)	
Stent migration	1 (1.3)	0 (0.0)	

ERCP: Endoscopic retrograde cholangio pancreatography.

On categorizing patients based on the type of SEMS deployed, 190 patients (78%) had uncovered SEMS while 54 patients (22%) had covered SEMS. Rates of PEP with uncovered and covered SEMS were 2.6% (5/190; mild, 4; moderate, 1) and 3.7% (2/54, both mild), respectively (P = 0.652). Mild bleeding occurred in 2 patients (1.1%) in the uncovered SEMS group alone. No significant differences were found between these two groups as to the incidence of post-ERCP complications (P = 1.000) (Table 5).

On comparing patients with no complications (n = 235) and those with complications (n = 9), the only

Table 4 Rates of complications on biliary stenting with limited endoscopic sphincterotomy according to location of pancreatic cancer *n* (%)

	Head *n* = 68	Body / Tail *n* = 11	*P* value
Post-ERCP complication type			1.000
Pancreatitis	1 (1.5)	0 (0.0)	
Mild/moderate	1 (1.5)	0 (0.0)	
Bleeding, mild	1 (1.5)	0 (0.0)	
Perforation	1 (1.5)	0 (0.0)	
Post-ERCP hyperamylasemia	8 (11.8)	2 (18.2)	0.624
Stent complication			1.000
None	60 (88.2)	10 (90.9)	
Stent occlusion	8 (11.8)	1 (9.1)	
Stent migration	0 (0.0)	0 (0.0)	

ERCP: Endoscopic retrograde cholangio pancreatography.

Table 5 Rates of complications on biliary stenting with limited endoscopic sphincterotomy according to stent type *n* (%)

	Uncovered *n* = 190	Covered *n* = 54	*P* value
Normal	181 (95.3)	49 (90.7)	
Abnormal	9 (4.7)	5 (9.3)	
Post-ERCP complication			1.000
Present	7 (3.7)	2 (3.7)	
Absent'	183 (96.3)	52 (96.3)	
Post-ERCP complication type			0.838
Pancreatitis	5 (2.6)	2 (3.7)	
Mild / moderate	4 (2.1) / 1 (0.5)	2 (3.7) / 0 (0)	
Bleeding, mild	2 (1.1)	0 (0)	
Perforation	0 (0)	0 (0)	
Post-ERCP hyperamylasemia	25 (13.2)	5 (9.3)	0.638
Stent complication			0.758
None	156 (82.1)	43 (79.6)	
Stent occlusion	33 (17.4)	11 (20.4)	
Stent migration	1 (0.5)	0 (0)	
Patency			0.012
No further procedure	156 (82.1)	43 (79.6)	
ERBD restent	1 (0.5)	0 (0)	
SEMS restent	28 (14.7)	4 (7.4)	
PTBD	5 (2.6)	7 (13)	

SEMS: Self-expandable metal stent; ERBD: Endoscopic retrograde biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; ERCP: Endoscopic retrograde cholangio pancreatography.

factor that was significantly different between the two groups was the number of SEMS initially deployed [one SEMS *vs* two SEMS (bilateral), $P = 0.031$] (Table 6). Of the 231 patients with one SEMS, 5 patients developed PEP and 2 patients developed mild bleeding, while of the 13 patients with two SEMS, 2 patients developed PEP.

DISCUSSION

ES is an established technique and is commonly used to facilitate biliary stone removal. In contrast, the role of routine ES prior to stent insertion is still controversial. Many endoscopists prefer to perform ES before stenting to reduce the risk of PEP, achieve better biliary drainage, and facilitate stent placement. However, sphincterotomy carries risks such as bleeding, perforation and pancreatitis[9]. Some studies have reported that the risks of ES might exceed any benefits owing to a high incidence of ES-related complications[15]. Cotton *et al*[20] reported bleeding and pancreatitis as major early complications with ES. Freeman *et al*[15] evaluated early complications following ES and reported their incidence as 9.8% (pancreatitis, 5.4%; bleeding, 2.0%).

Previous studies, however, lack details regarding the extent of ES and its correlation with the incidence of complications. In this study, we performed limited ES before SEMS placement and described the safety of limited ES by evaluating early post-ERCP complications in patients with malignant biliary obstruction. The overall rate of early post-ERCP complications after SEMS placement with limited ES was 3.7%, including a 2.9% incidence of PEP, and 0.8% of mild bleeding. These

Table 6 Characteristics according to complications on biliary stenting with limited endoscopic sphincterotomy *n* (%)

	No complication *n* = 235	Complication *n* = 9	*P* value
Age (yr), mean ± SD (range)	70.61 ± 10.33	75.27 ± 5.55	0.993
Gender			1.000
Male	126 (53.6)	5 (55.5)	
Female	109 (46.6)	4 (44.5)	
Total bilirubin (mg/dL), pre-procedure, mean ± SD	7.00 ± 6.46	8.38 ± 6.54	0.362
Normal	52 (22.2)	1 (11.1)	0.453
Elevated	183 (77.8)	8 (88.9)	
Hyperamylasemia, pre-procedure	13 (5.5)	1 (11.1)	0.485
Cholangitis, pre-procedure	64 (27.2)	4 (44.4)	0.472
Diagnosis			0.748
Cholangiocarcinoma	112 (47.6)	6 (66.7)	
Hilar	71 (30.2)	4 (44.4)	
Distal	41 (17.4)	2 (22.2)	
Pancreatic cancer	77 (32.8)	2 (22.2)	
Head	66 (28.1)	2 (22.2)	
Body/tail	11 (4.7)	0 (0.0)	
Gallbladder cancer	20 (8.5)	1 (11.1)	
Ampullary cancer	18 (7.7)	0 (0.0)	
Hepatocellular carcinoma	3 (1.3)	0 (0.0)	
Others	5 (2.1)	0 (0.0)	
Pancreatic duct invasion			0.324
Yes	80 (34.0)	5 (55.6)	
No	155 (66.0)	4 (44.4)	
Lymph node metastasis			0.176
Yes	166 (70.6)	5 (55.6)	
No	69 (29.4)	4 (44.4)	
Pancreatic duct injection			0.662
0	181 (77.0)	6 (66.7)	
1-2	23 (9.8)	1 (11.1)	
⩾ 3	31 (13.2)	2 (22.2)	
Number of inserted SEMS			0.031
1	224 (95.3)	7 (77.8)	
2	11 (4.7)	2 (22.2)	
Stent type			1.000
Uncovered	183 (77.9)	7 (77.8)	
Covered	52 (22.1)	2 (22.2)	
Stent complication			1.000
Stent occlusion	42 (17.9)	2 (22.2)	
Stent migration	1 (0.4)	0 (0.0)	
Patency			0.512
No further procedure	192 (81.7)	7 (77.8)	
ERBD restent	1 (0.4)	0 (0.0)	
SEMS restent	31 (13.2)	1 (11.1)	
PTBD	11 (4.7)	1 (11.1)	

SEMS: Self-expandable metal stent; ERBD: Endoscopic retrograde biliary drainage; PTBD: Percutaneous transhepatic biliary drainage; ERCP: Endoscopic retrograde cholangio pancreatography.

rates of complications are relatively low compared to the complication rates of approximately 10% and an overall mortality of 0.42% in published data[2,13-17].

Bleeding is a serious complication of ES and its incidence is reported to be between 1 and 10%[12,22-25]. In our study, only 2 patients (0.8%) developed mild bleeding, which could be managed by conservative treatment. No instances of moderate or severe bleeding were reported. Wang *et al*[26], in their analysis of delayed hemorrhage following ES in 1741 patients did not find delayed bleeding in any patient who underwent small ES (*n* = 194). These results might be related to the limited extent of the ES and the compressive effect of the SEMS[27]. On the basis of these studies, limited ES does not seem to be associated with clinically significant bleeding.

A recent meta-analysis by Cui *et al*[28], analyzing biliary stenting for malignant biliary obstruction reported that the incidence of PEP was significantly lower with ES than without ES (3.5% *vs* 8.9%, *P* = 0.04, OR = 0.34, 95%CI: 0.12-0.93) and recommended ES before stent placement as a useful option to reduce the incidence of PEP. Similar low rates (2.2%) were reported by Giorgio *et al*[29] in their randomized control trial involving 10 Fr plastic stent after ES for inoperable malignant common bile duct (CBD) obstruction. In our study, despite the absence of a control group, the low rate of PEP (2.9%) is comparable to the results of the

two above-mentioned studies. Our PEP rates are also low compared to the rates of 9.4% and 6.3%, with metal stent placement following ES for distal biliary strictures, reported by Hayashi *et al*[17] and by Kahaleh *et al*[30], respectively. In our study, the incidence of PEP was 4.0% in hilar cholangiocarcinoma and 2.3% in distal cholangiocarcinoma. Although the outcomes with ES for malignant biliary strictures, especially cholangiocarcinoma, are controversial, several previous studies have demonstrated a lower incidence of PEP in the ES group compared to the non-ES group[8,9,31]. Jeong *et al*[8] investigated the risk of pancreatitis in patients with malignant obstructive jaundice following percutaneous or transpapillary stent placement. They also studied the effect of preliminary ES in the transpapillary stent group. Their results demonstrated a higher rate of pancreatitis in the transpapillary stent group ($P = 0.502$) and the authors concluded that SEMS placement through the intact sphincter of Oddi may increase the risk pancreatitis.

The management of hilar obstruction is more difficult than distal bile duct strictures because of the underlying anatomical and technical complexity. Bilateral stent placements for Bismuth type Ⅱ to Ⅳ hilar cholangiocarcinoma are also very complicated and result in increased endoscopic manipulations[32]. The higher incidence of post-ERCP complications in patients who had two SEMS (bilateral stents) placed could be related to these reasons. In these situations, limited ES before stenting could be an effective strategy for facilitating more complex stenting procedures[33]. Limited ES may allow for easier stent placement and reduce resistance to biliary instrumentation. Additionally, proximal bile duct strictures may contribute to a fulcrum effect resulting in medial displacement of the distal stent and, consequently, stent related compression of the pancreatic duct[9]. Limited ES might prevent the risk of pancreatitis by reducing stent-related pancreatic duct obstruction. In case of distal CBD strictures, ES may allow the stent to achieve a better final diameter, and thus, better drainage.

Our data demonstrated a lower rate of PEP in patients with cholangiocarcinoma compared to previous studies. Limited ES, therefore, could be an effective and useful technique to prevent PEP following stenting for cholangiocarcinoma, especially hilar tumors. The incidence of PEP was lesser in pancreatic cancers than in cholangiocarcinoma in this study (1.3% *vs* 3.4%, $P = 0.681$). Some studies demonstrated that pancreatic cancers with obstruction of the main pancreatic duct had a lower degree of PEP, possibly due to diminished pancreatic exocrine function and suggested that ES may be unnecessary in such cases[12,17,28,32]. Although further confirmation is required, we noted that performing limited ES prior to SEMS placement in patients with unresectable pancreatic cancers did not result in a higher incidence of adverse events compared to published data[17]. Additionally, it is possible that ES may be advantageous in selected cases, depending on pancreatic duct status, stent diameter, stent type (especially fully covered SEMS) or ampulla size, in rendering the procedure easier as biliary strictures secondary to pancreatic cancer tend to be narrow and rigid. ES may also facilitate stent exchange during the follow-up period[32] as demonstrated in our study, where the success rate of SEM restenting, when indicated, was 97%.

Stent migration is a late complications of biliary stenting with ES. Stent migration seems to be associated with stent type as well as ES. Covered SEMS are not fully embedded in bile duct, and therefore, are associated with the potential risk of stent migration. A previous study reported increased frequency of stent migration when ES was performed before placement of covered SEMS[16]. In contrast, other studies did not support this finding[29,34]. In our study, stent migration occurred in only 1 patient (0.4%) and limited ES did not seem to be a significant factor associated with migration, regardless of the stent type.

This retrospective study has a few limitations. First, the possibility of inaccurate data collection cannot be overlooked. For example, procedure-related abdominal pain is difficult to distinguish from the breakthrough pain of malignancy and may have contributed to a bias in measuring the rate of PEP. Second, this study had a single-center design without a control group (non-ES group), which might influence the interpretation of the effect of limited ES. Further prospective multicenter studies with the inclusion of control groups are needed to overcome these limitations.

In conclusion, limited ES is a feasible, safe and effective procedure to facilitate placement of SEMS in patients with malignant biliary obstruction. Limited ES is not significantly associated with complications like severe bleeding or PEP and its use may represent a better strategy to achieve successful stent placement, especially in cases like hilar strictures that require complex procedural techniques.

COMMENTS

Background

Endoscopic biliary stent placement has become the primary management therapy for palliation in patients with malignant biliary obstruction. Endoscopic sphincterotomy (ES) is performed to reduce the risk of post-ERCP pancreatitis (PEP) and facilitate stent placement. Although many endoscopists routinely perform ES before self-expandable metal stent (SEMS) placement, the role of ES is still controversial in biliary stenting. Effects and complications on the degree of ES also need to be investigated. There have been few studies on the complications or effects of limited ES.

Research frontiers

At present, there have been some reports to evaluate the safety and efficacy of ES before placement of SEMS and the existing data is contradictory. Currently, there are no guidelines regarding ES for biliary stenting. There is a lack of detail, regarding the extent of ES, and its correlation with complications.

Innovations and breakthroughs

Limited ES is not significantly associated with complications like severe

bleeding or PEP. It may be useful to achieve successful stent placement. Limited ES is a feasible, safe and effective procedure to facilitate placement of SEMS in patients with malignant biliary obstruction.

Applications

This retrospective study showed that limited ES could be useful to facilitate placement of SEMS, especially in cases, like hilar strictures, requiring complex procedural techniques without major complications. Further large randomized controlled trials are required.

Terminology

ES is a method to provide access to the biliary system for therapy, which means cutting of the sphincter or muscle that lies at the juncture of the intestine with both the bile and pancreatic ducts. Limited ES is defined as sphincterotomy less than one-third the extent of major ES.

Peer-review

This is an interesting manuscript that has not been published extensively. The authors showed in this study that the clinical outcomes in patients who did undergo limited ES before placement of SEMS for malignant biliary obstruction. The results provide new evidence that limited ES could be a feasible strategy for SEMS placement without significant complications.

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ORIGINAL ARTICLE

Retrospective Study

Fibrin sealant for closure of mucosal penetration at the cardia during peroral endoscopic myotomy: A retrospective study at a single center

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Author contributions: Zhang WG analyzed the data and wrote the manuscript; Li HK acquired the data; Linghu EQ designed the research.

Institutional review board statement: The study was carried out under the ethics committee approval from the Chinese PLA General Hospital (Beijing China).

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

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Abstract

AIM

To assess the efficacy and safety of fibrin sealant for closure of mucosal penetration at the cardia during peroral endoscopic myotomy (POEM).

METHODS

Twenty-four patients who underwent POEM and experienced mucosal injury of the cardia during the procedure were retrospectively identified. Of the 24 patients, 21 had mucosal penetration and 3 had only slight mucosal damage without penetration. The 21 patients with mucosal penetration received fibrin sealant for closure at the site of penetration. Penetration-related characteristics, treatment, and recovery were reviewed for all 21 patients to assess the efficacy and safety of fibrin sealant for closure of mucosal penetration at the cardia. Clinical data, including general characteristics, procedure-related parameters, Eckardt scores, lower esophageal sphincter pressures (LESP), and esophagogastroduodenoscopy (EGD) results, were analyzed to determine their influence on treatment success after mucosal penetration during POEM.

RESULTS

All 21 patients had a solitary mucosal penetration in the cardia (12 in esophageal region of the cardia, 9 in the stomach region of the cardia, and 1 in both the esophageal and stomach regions). Twelve had a

hole-like penetration and 9 had a linear penetration. For those with a hole-like penetration, the mean size was 0.14 cm^2 (0.02-0.32 cm^2). For those with a linear penetration, the median size was 0.37 cm (0.10-1.00 cm). Closure of the mucosal penetration using fibrin sealant was performed successfully in all 21 patients (two patients required 5 mL fibrin sealant, and the remaining 19 patients required 2.5 mL). Two patients had a nasogastric tube placed for five days after POEM; the remaining 19 patients were kept fasting for 3 d. All 21 patients were discharged after a median of 5 d (range: 5-7 d) postoperatively. During a median 42 mo (range: 9-62 mo) follow-up, all 21 patients with a mucosal penetration successfully healed without the occurrence of infection, ulcer, or esophagitis. Furthermore, the median LESP decreased from 31.9 mmHg (range: 21.9-67.1 mmHg) preoperatively to 20.3 mmHg (range: 6.0-41.0 mmHg) postoperatively ($P < 0.05$). The median preoperative and postoperative Eckardt scores were 5.0 (range; 4-10) and 1.0 (range: 0-4), respectively ($P < 0.05$). Of the 21 patients with mucosal penetration, symptom remission, which is defined as a postoperative Eckardt score $\leqslant 3$, was achieved in 20 patients (95.2%) indicating that mucosal penetration did not influence the success of POEM treatment if closed successfully using fibrin sealant.

CONCLUSION

Fibrin sealant is safe and effective for closure of mucosal penetration during POEM. Mucosal penetrations do not appear to influence the treatment success of POEM if closed successfully using fibrin sealant. Additional studies regarding the feasibility, efficacy, and safety of fibrin sealant for closure of larger mucosal penetrations is warranted.

Key words: Fibrin sealant; Mucosal penetration; Peroral endoscopic myotomy; Efficacy; Safety

Core tip: Mucosal penetration is one of the most dangerous adverse events during peroral endoscopic myotomy (POEM). We first reported the feasibility of fibrin sealant for closure of mucosal penetration at the cardia in two cases in 2012. However, there remains a lack of evidence about the treatment response to fibrin sealant for mucosal penetration in a cohort of patients who experienced this complication. Thus, we retrospective identified and analyzed the cases for 21 patients who experienced a mucosal penetration and received fibrin sealant for penetration closure during POEM, providing further support for the efficacy and safety of fibrin sealant for penetration closure. Moreover, instructions regarding the usage of fibrin sealant for penetration closure were provided for endoscopists who might be worried about mucosal penetrations during POEM.

Zhang WG, Linghu EQ, Li HK. Fibrin sealant for closure of mucosal penetration at the cardia during peroral endoscopic myotomy: A retrospective study at a single center. *World J Gastroenterol* 2017; 23(9): 1637-1644 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1637.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1637

INTRODUCTION

Peroral endoscopic myotomy (POEM) is an effective and safe procedure for achalasia, and is becoming one of the first-line therapies to treat achalasia[1-4]. However, some major perioperative adverse events after POEM have been reported; mucosal penetration is one of the most dangerous adverse events[1,2,5,6]. Mucosal penetration has been reported to occur in 4.2%-17.3% of POEM procedures[1,2,5,7]. Different studies have reported on different treatment strategies, including observation without special treatment, sealing of the penetration injury by hemostatic clips, and closing the penetration defect using fibrin sealant[8,9]. Mucosal penetration usually occurs at the cardia, where a myotomy is performed during POEM. We previously reported the usage of fibrin sealant for mucosal penetration at the cardia in two cases in 2012[7]. However, long-term outcomes with a larger population are needed to further assess this treatment strategy. To the best of our knowledge, there is still no evidence regarding the treatment response to fibrin sealant for mucosal penetration during POEM in a larger cohort. The purpose of the present study was to evaluate the efficacy and safety of fibrin sealant for closure of mucosal penetration at the cardia during POEM.

MATERIALS AND METHODS

Patients

Twenty-four patients who underwent POEM and experienced mucosal injury of the cardia during the procedure between November 2010 and February 2016 were identified and collected. Of these, 21 had mucosal penetration and 3 had only minor mucosal damage without penetration. All 21 patients with a penetrating injury received fibrin sealant for closure of the mucosal penetration; these 21 patients were included in the analysis.

Prior to undergoing POEM, all patients had undergone esophagogastroduodenoscopy (EGD), high-resolution manometry (HRM), and had their symptoms evaluated using Eckardt scores to confirm a diagnosis of achalasia.

POEM procedure

Patients were admitted and fasted for 48 h before

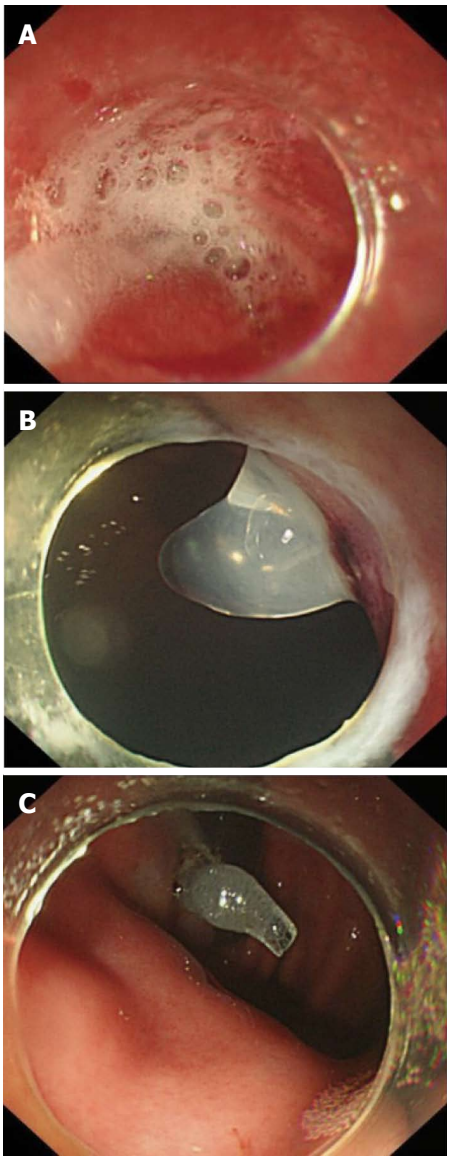

Figure 1 Closure of the mucosal penetration using fibrin sealant. A: Spraying fibrin sealant in the submucosal tunnel; B: Fibrin sealant fully covers the penetration (imaging from esophageal lumen); C: Fibrin sealant fully covers the penetration (imaging from stomach lumen).

POEM. Patients underwent EGD prior to POEM to ensure that there was no residual food in the esophageal lumen. During the procedure, patients were kept in a supine position with the right shoulder elevated, and general anesthesia was administered with continuous monitoring of electrocardiography (ECG), respirations, blood pressure, and oxygen saturation.

An additional cap attached at the top of the gastroscopy was required. With the outside cap diameter (12.0 mm) as reference, the penetration size was estimated. Then, POEM was performed. First, a submucosal injection was performed with methylene blue saline solution (1:10000), and a mucosal incision was made at the right posterior esophageal wall approximately 6-10 cm from the gastroesophageal junction (GEJ). Then, a submucosal tunnel was established, passing over the GEJ to approximately 2-3 cm into the proximal stomach. The myotomy started 2 cm distal to the incision and extended 2-3 cm into the stomach. After complete hemostasis and ensuring that an endoscope could easily pass the cardia, the mucosal incision was sutured with hemostatic clips.

In the present study, four different types of myotomy were performed, including inner circular muscle myotomy, full-thickness myotomy, glasses-style anti-reflux myotomy, and progressive full-thickness myotomy.

Glasses-style anti-reflux myotomy retains about 1 cm of longitudinal muscle at the level of the dentate line after incision of the inner circular muscle, and makes selective incision of the longitudinal muscle right above and below the dentate line. The retained 1 cm of longitudinal muscle is expected to achieve the best result to prevent reflux after POEM.

Closure of mucosal penetration

Once mucosal penetration occurred during the POEM procedure, fibrin sealant was sprayed into the penetrating injury in the submucosal tunnel under direct endoscopic visualization to ensure that the fibrin sealant fully covered the defect (Figure 1). The amount of fibrin sealant consumed was based on the size of the defect. For large penetrations, which were difficult to close only using fibrin sealant, a hemostatic clip was used to make a preliminary clipping that approximated the edges of the defect; then, fibrin sealant was sprayed to fully cover the penetration defect (Figure 2).

Postoperative treatment

X-ray or chest and abdomen computed tomography (CT) was routinely performed postoperatively to evaluate for gas-related complications immediately after POEM. Delayed hemorrhage, pulmonary infection, and other complications were also monitored under EGD after the procedure. Evaluation of tunnel infection or penetration-raised esophagitis also occurs during the postoperative EGD examination, especially if the mucosa was penetrated during the procedure. After fasting for 3 d postoperatively, a liquid diet was followed for 1 d, then a soft diet. A regular diet was resumed 1 mo after POEM. Postoperative medications, including double-dose proton pump inhibitor (PPI) and antibiotics, were prescribed; PPI was required for at least 4 wk.

Follow-up

Patients were scheduled for a follow-up visit at 3 mo, 6 mo, 1 year, and 2 years after POEM. EGD, high-resolution manometry, and 24-h esophageal pH monitoring were required at the follow-up to assess the healing of the mucosal penetration or the entry incision, lower esophageal sphincter pressures, and postoperative esophagitis, respectively. For patients who experienced mucosal penetration during POEM,

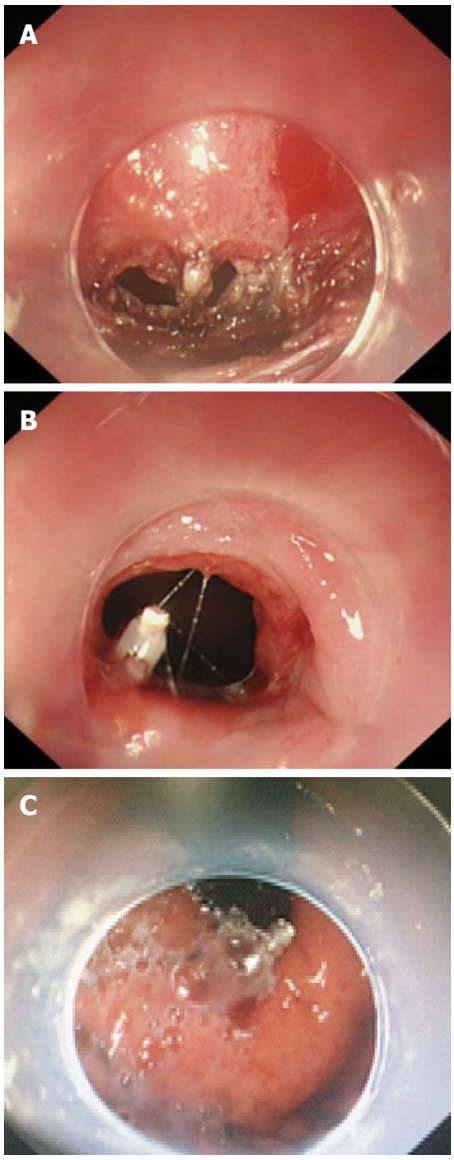

Figure 2 Closure of a 0.8 cm × 0.4 cm mucosal penetration using a hemostatic clip and fibrin sealant. A: The appearance of the 0.8 cm × 0.4 cm mucosal penetration (imaging from the submucosal tunnel); B: A hemostatic clip was used to make a preliminary clipping (imaging from esophageal lumen); C: Fibrin sealant fully covers the preliminary clipped penetration (imaging from stomach lumen).

two additional follow-ups at one week and six weeks postoperatively were added. Postoperative complications and Eckardt scores for each patient were recorded *via* the telephone. Treatment success was defined as Eckardt scores no greater than 3.

Statistical analysis

All statistical analyses were performed using SPSS software version 17.0. Variables are expressed as mean or median. Paired-samples Student's *t*-test or Wilcoxon matched-pairs signed-ranks test was used to estimate the treatment outcomes of POEM. All reported *P*-values are two-tailed; *P*-values of < 0.05 were considered statistically significant.

Table 1 Clinical characteristics and procedure-related parameters for 21 consecutive patients who experienced mucosal penetration during peroral endoscopic myotomy procedure

Patient characteristics	
Sex, female/male (*n*)	12/9
Age (yr), mean (range)	38.0 (15-64)
Symptom duration (mo), median (range)	26.0 (10-360)
Previous treatment (*n*)	
Botox injection	3
Bouginage	1
Chicago classification (*n*)	
Type Ⅰ	2
Type Ⅱ	18
Type Ⅲ	1
Procedure-related parameters	
Procedure time (min.), median (range)	58.9 (20.0-141.0)
Tunnel length (cm), mean (range)	11.7 (7-18)
Myotomy length (cm), mean (range)	5.6 (3-10)
Myotomy type (*n*)	
Inner circular muscle myotomy	10
Full-thickness myotomy	1
Glasses-style anti-reflux myotomy	1
Progressive full-thickness myotomy	9

Table 2 Characteristics of the 21 mucosal penetrations and the treatment outcomes using fibrin sealant

Penetration shape, *n* (%)	
Hole-like penetration	12 (57.1)
Linear penetration	9 (42.9)
Penetration location	
Esophageal part of cardia	12 (61.9)
Stomach part of cardia	8 (38.1)
Both esophageal and stomach parts of cardia	1 (4.8)
Penetration size	
Hole like penetration (cm^2), mean (range)	0.14 (0.02-0.32)
Linear penetration (cm), median (range)	0.37 (0.10-1.00)
Consumed fibrin sealant amount (*n*)	
5.0 mL	3
2.5 mL	18
Postoperative treatment	
Placement of nasogastric tube (*n*)	2
Postoperative stay (d), median (range)	5 (5-7)

RESULTS

Patient characteristics and procedure-related parameters

As shown in Table 1, the study cohort consisted of 9 men and 12 women, aged 15 to 64 years (mean, 38.0 years). Among the 21 patients with mucosal penetration, the median duration of symptoms was 26.0 mo (range, 10-360 mo). Three patients had a previous Botox injection, and one had a previous bouginage. According to the Chicago classification, 2 patients were classified as type Ⅰ, 18 as type Ⅱ, and 1 as type Ⅲ. All 21 patients successfully underwent POEM with a median operative time of 58.9 min (range, 20.0-141.0 min). The mean length of the submucosal tunnel and myotomy was 11.7 cm (range, 7-18 cm) and 5.6 cm

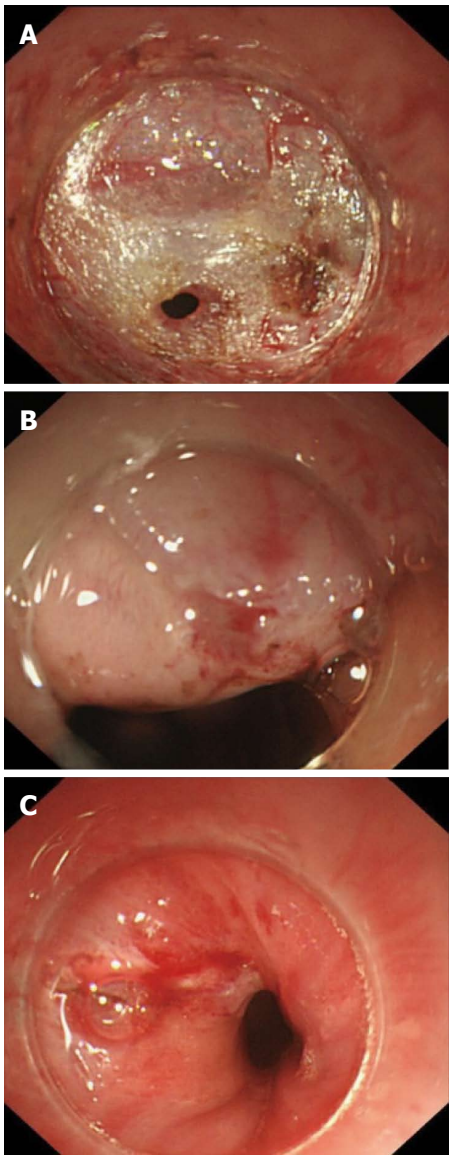

Figure 3 Two kinds of mucosal penetration under esophagogastroduodenoscopy. A: Hole-like penetration (imaging from submucosal tunnel); B: Hole-like penetration (imaging from esophageal lumen); C: Linear penetration (imaging from esophageal lumen).

(range, 3-10 cm), respectively.

With regards to myotomy type, 10 patients received an inner circular muscle myotomy, 9 had a progressive full-thickness myotomy, 1 had a full-thickness myotomy, and 1 had a glasses-style anti-reflux myotomy.

Mucosal penetration characteristics and treatment outcomes after fibrin sealant

As shown in Table 2, all 21 patients had a solitary mucosal penetration in the cardia (12 in the esophageal region of cardia, 9 in the stomach region of cardia, and 1 in both the esophageal and stomach regions). Twelve patients had a hole-like penetration, while 9 had a linear penetration (Figure 3). Among those with a hole-like penetration, the mean size was 0.14 cm^2 (range, 0.02-0.32 cm^2). For the linear penetrations,

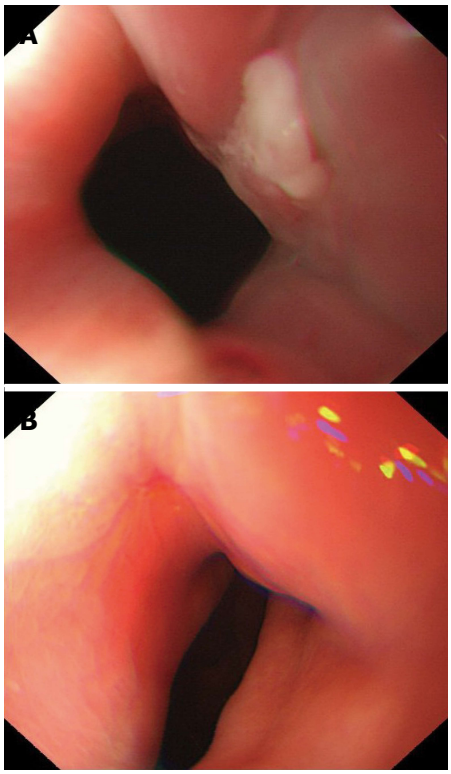

Figure 4 Healing process of the mucosal penetration after being closed using fibrin sealant. A: The appearance of penetration at one week after peroral endoscopic myotomy (POEM) (imaging from esophageal lumen); B: The appearance of penetration at six weeks after POEM (imaging from esophageal lumen).

the median size was 0.37 cm (range, 0.10-1.00 cm). Closure of mucosal penetration using fibrin sealant was performed successfully in all 21 patients. Two cases, one of which was a relatively longer linear penetration (1.0 cm), consumed 5 mL of fibrin sealant. Only 2.5 mL of sealant was required for the remaining 19 cases. One case had a 0.8 cm × 0.4 cm hole-like penetration that was difficult to close using only fibrin sealant; thus, one hemostatic clip was used to make a preliminary closure of the penetration, and fibrin sealant was then sprayed to fully cover the mucosal defect. The first two patients to experience mucosal penetration had a nasogastric tube placed for 5 d after POEM; the remaining 19 patients were kept fasting for 3 d. The mucosal penetrations had an appearance on EGD at one week postoperatively as shown in Figure 4A and an appearance at 6 wk as shown in Figure 4B. All 21 patients were discharged after a median of 5 d (range, 5-7 d) postoperatively. During a median 42 mo (range, 9-62 mo) follow-up, all 21 mucosal penetrations successfully healed without the occurrence of penetration-raised tunnel-infection, ulcer, esophagitis, mediastinal leak, or peritoneal leak. Detailed data of the 21 patients with mucosal penetrations are shown in Table 3.

Treatment outcomes of POEM and complications

Symptom remission, which was defined as postopera-

Table 3 Detailed data of the mucosal penetrations from all 21 patients

Number	Shape	Location	Estimated size (cm/cm^2)	Postoperative treatment	Postoperative stay (d)	Amount of consumed fibrin sealant (mL)	Postoperative complaint
1	Hole like	GOC	0.4 × 0.4	NG tube	7	5	Slight abdominal pain
2	Hole like	GOC	0.4 × 0.5	NG tube	7	2.5	Normal
3	Hole like	GOC	0.3 × 0.2	Fasting	7	2.5	Normal
4	Linear	EOC	0.3	Fasting	7	2.5	Normal
5	Hole like	EOC	0.4 × 0.3	Fasting	7	2.5	Normal
6	Linear	EOC	0.3	Fasting	6	2.5	Normal
7	Linear	EOC	0.1	Fasting	6	2.5	Normal
8	Linear	GOC	0.4	Fasting	6	2.5	Normal
9	Linear	GOC	0.4	Fasting	6	2.5	Normal
10	Hole like	EOC	0.3 × 0.2	Fasting	5	2.5	Normal
11	Linear	EOC	0.2	Fasting	5	2.5	Normal
12	Hole like	EOC	0.2 × 0.2	Fasting	5	2.5	Normal
13	Linear	BOC	1.0	Fasting	5	5	Normal
14	Hole like	EOC	0.8 × 0.4	Fasting	5	2.5 (one hemostatic clip)	Normal
15	Linear	EOC	0.3	Fasting	5	2.5	Normal
16	Hole like	GOC	0.5 × 0.5	Fasting	5	2.5	Normal
17	Hole like	EOC	0.4 × 0.4	Fasting	5	2.5	Normal
18	Hole like	GOC	0.3 × 0.3	Fasting	5	2.5	Normal
19	Hole like	EOC	0.4 × 0.4	Fasting	5	2.5	Normal
20	Linear	GOC	0.3	Fasting	5	2.5	Normal
21	Hole like	EOC	0.1 × 0.2	Fasting	5	2.5	Normal

NG tube: Nasogastric tube; GOC: Gastric part of the cardia; EOC: Esophageal part of the cardia; BOC: Both gastric and esophageal parts of the cardia.

Table 4 Symptom relief, manometry outcomes, and reflux complications of the 21 patients who experienced mucosal penetration during peroral endoscopic myotomy

Follow-up period (mo), median (range)	42.0 (9-62)
Symptom relief	
Eckardt score, median (range)	
Pre-treatment	5.0 (4-10)
Post-treatment	1.0 (0-4)
Pre/post-treatment difference value	4.8 (1-9)
Treatment success (Eckardt score ⩽ 3), *n* (%)	20 (95.2)
Manometry outcomes	
Manometry follow-up rate, *n* (%)	15 (71.4)
LESP (mmHg), median (range)	
Pre-treatment	31.9 (21.9-67.1)
Post-treatment	20.3 (6.0-41.0)
Pre/post-treatment difference value	14.1 (9.6-35.2)
Post-POEM esophagitis on EGD	
LA-A	1
LA-B	2
Overall, *n* (%)	3 (14.3)
Gas-related complications, *n*	
Pneumothorax	1
Pneumoperitoneum	1
Pneumomediastinum	1
Overall	3

POEM: Peroral endoscopic myotomy; EGD: Esophagogastroduodenoscopy; LA-A: Los Angeles classification A; LA-B: Los Angeles classification B; LESP: Lower esophageal sphincter pressure.

tive Eckardt score ⩽ 3, was achieved in 20 patients (95.2%) during a median of 42 mo (range, 9-62 mo) follow-up (Table 4). The median preoperative and postoperative Eckardt score were 5.0 (range, 4-10) and 1.0 (range, 0-4), respectively ($P < 0.05$). A total of 15 patients had HRM both before and after treatment; 6 patients did not undergo post-operative HRM due to procedure-related discomfort or for other personal reasons. The median lower esophageal sphincter (LES) pressure decreased from 31.9 mmHg (range, 21.9-67.1 mmHg) preoperatively to 20.3 mmHg (range, 6.0-41.0 mmHg) postoperatively ($P < 0.05$), indicating a statistically significant decrease after POEM. All the treatment outcomes, mentioned above, indicated that mucosal penetration did not influence the treatment success of POEM if the defect was closed successfully using fibrin sealant.

In terms of complications, 3 patients had post-POEM esophagitis on EGD; 2 were classified as Los Angeles classification B and one as Los Angeles classification A. Moreover, another 3 patients had gas-related complications: 1 experienced pneumothorax, 1 experienced pneumoperitoneum, and 1 experienced pneumomediastinum.

DISCUSSION

Esophageal achalasia is an esophageal motility disorder of unknown cause and is characterized by failure of the LES to relax and impaired peristalsis of the esophageal body[10]. Conventional therapies for achalasia include pharmacological therapy, endoscopic balloon dilation, and Heller-Dor surgery. With recent advances in endoscopic treatment techniques and devices, Inoue *et al*[9] have developed peroral endoscopic myotomy, in which the myotomy is performed through a submucosal tunnel. Excellent long-term outcomes after POEM have been reported[1,2], and POEM is expected to become a first-line therapy for achalasia

requiring surgical intervention. However, some major perioperative adverse events from POEM have also been reported, with mucosal penetration being one of the most dangerous adverse events[1,2,5,6]. Mucosal penetration during POEM occurs at a rate ranging from 4.2%-17.3% depending on the study[1,2,5,7]. Treatment for this complication has varied, with some patients undergoing observation without special treatment, being sealed by multiple clips or an endoscopic suture device (OverStitch™ Endoscopic Suturing System; Apollo Endosurgery Austin, Texas), or being treated with the defect being closed using fibrin sealant[11-15]. Closure using hemostatic clips is not an ideal method. Once target mucosa is clipped, adjacent mucosa has the tendency to spontaneously split, making it hard to completely seal the penetration. Using endoscopic suture with the OverStitch system is usually considered when the mucosal penetration is large and difficult to close using conventional clips.

We first reported the usage of fibrin sealant for closure of mucosal penetration at the cardia in two cases in 2012[7]. The present study further supports the efficacy and safety of fibrin sealant for closure of mucosal penetration, including long-term follow-up in a larger population (21 patients). The biggest risk of mucosal penetration is that the fluids from the stomach or the esophagus could flow into the submucosal tunnel or the mediastinum and cause tunnel-infection, ulceration, esophagitis, mediastinal leak, or peritoneal leak[15,16]. In our study, all 21 mucosal penetrations healed successfully without tunnel-infection, ulceration, esophagitis, mediastinal leak, or peritoneal leak occurring. Of note, all 21 mucosal penetrations in the present study occurred at the cardia; one explanation for this might be that the small operating space and abundant submucosal vessels that demand repeated electrocoagulation during the POEM procedure make the mucosa in this area vulnerable. We presented the healing process of the mucosa after being closed using fibrin sealant (Figure 4). During a median 42 mo (range, 9-62 mo) follow-up, all patients had completely healed.

The required amount of fibrin sealant to adequately cover the mucosal injury was based on the size of the penetration; the endoscopist must ensure that the penetration is fully covered. In this cohort, we utilized 5 mL of fibrin sealant in the first patient with penetration because of lack of experience using this technique. Another patient who had a longer linear penetration (1.0 cm) consumed 5 mL fibrin sealant. The remaining 19 cases only required 2.5 mL. Of note, for penetration injuries that create larger defects, which are difficult to close using only fibrin sealant, it is suggested that one or two hemostatic clips be used to make a preliminary clipping that approximates the edges of the mucosal defect before then using fibrin sealant to fully cover the hole. Nasogastric tubes were placed postoperatively in the first two cases with penetration, again due to lack of experience with patient recovery from this technique. However, the remaining 19 cases did not require a nasogastric tube and had excellent healing results, suggesting that the postoperative placement of a nasogastric tube is not necessary in cases with a relatively small penetration.

The treatment outcomes of POEM for the 21 patients with mucosal penetration were excellent, with a 95.2% treatment success (Eckardt score $\leqslant$ 3), a 14.3% rate of gas-related complications, and a 14.3% rate of post-POEM esophagitis, indicating that mucosal penetration did not influence the treatment success of POEM if closed successfully using fibrin sealant.

Given that the sizes of the mucosal penetrations in this study were all relatively small, it is not clear whether the defects could have been observed and would have closed spontaneously. Therefore, a prospective randomized controlled trial comparing observation without special treatment to treatment with fibrin sealant is warranted. In previous studies evaluating intraoperative mucosal penetration during POEM, the injured mucosa could be closed only by prolonged fasting in those who received inner circular muscle myotomy. In the present study, 10 patients had an inner circular muscle myotomy, 1 had a full-thickness myotomy, 1 had a glasses-style anti-reflux myotomy, and 9 had a progressive full-thickness myotomy. Further research is needed to determine if the injured mucosa was more likely to close spontaneously in those who received inner circular muscle myotomy than in those who received a full-thickness myotomy. Our study is not without limitations. One limitation was that the submucosal defects in our study were all relatively small, so we cannot draw conclusions regarding the feasibility, efficacy, and safety of fibrin sealant in closing large mucosal penetrations. Additionally, with a small sample size of only 21 patients, we were not able to stratify our results to draw conclusions regarding the required amount of fibrin sealant based on the penetration size. Another limitation is that this was a single center study, suggesting that our results may not be representative of findings in other hospitals. However, to the best of our knowledge, this is the largest published study regarding the treatment response to fibrin sealant for mucosal penetration during POEM, incorporating data from 21 patients. The present study also provides instruction regarding the usage of fibrin sealant for penetration closure for endoscopists, which may be especially helpful for those who are unfamiliar with the technique or who might be worried about mucosal penetrations during POEM.

In conclusion, the use of fibrin sealant to close mucosal penetration during POEM is safe and effective. Mucosal penetrations do not appear to influence the treatment success of POEM if closed successfully using fibrin sealant. However, further research regarding the feasibility, efficacy, and safety of fibrin sealant for closing larger mucosal penetrations is warranted.

COMMENTS

Background

Peroral endoscopic myotomy (POEM) has been proved to be an effective and safe procedure for achalasia. However, mucosal penetration has been reported to be one of the most dangerous adverse events during POEM.

Research frontiers

The treatments for the injured mucosa include observation without special treatment, sealed by multiple clips hemostatic clips, endoscopic suture device (OverStitch™ Endoscopic Suturing System; Apollo Endosurgery Austin, Texas) or closed using fibrin sealant. Fibrin sealant seems to be effective and safe for the penetration closure and the authors have reported a case in 2012. However, there is still no evidence regarding the treatment response to fibrin sealant for mucosal penetration during POEM in a larger cohort.

Innovations and breakthroughs

To the best of our knowledge, this is the largest published study regarding the treatment response to fibrin sealant for mucosal penetration during POEM, incorporating data from 21 patients.

Applications

The present study provided an instruction about the usage of fibrin sealant for penetration closure for endoscopists, especially for novices, who might be worried about the mucosal penetrations during POEM.

Terminology

POEM: Peroral endoscopic myotomy, a recently developed endoscopic therapeutic technique, was performed for achalasia. peroral endoscopic myotomy.

Peer-review

This is an interesting, retrospective study from one center, on fibrin sealant for closure of cardia mucosal penetration during POEM.

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ORIGINAL ARTICLE

Clinical Trials Study

Outcomes of gastrointestinal defect closure with an over-the-scope clip system in a multicenter experience: An analysis of a successful suction method

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Institutional review board statement: This study was reviewed and approved by the Ethics Committee of the Kagawa Medical University Hospital and each institution.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent. For full disclosure, the details of the study are published on the home page of the Kagawa Medical University Hospital and each institution.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at kobara@med.kagawa-u.ac.jp. Informed consent form participants for data sharing was not obtained but the presented data are anonymized and risk of identification is low.

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Abstract

AIM

To demonstrate the clinical outcomes of a multicenter experience and to suggest guidelines for choosing a suction method.

METHODS

This retrospective study at 5 medical centers involved 58 consecutive patients undergoing over-the-scope clips (OTSCs) placement. The overall rates of technical success (TSR), clinical success (CSR), complications, and procedure time were analyzed as major outcomes. Subsequently, 56 patients, excluding two cases that used the Anchor device, were divided into two groups: 14 cases of simple suction (SS-group) and 42 cases using the Twin Grasper (TG-group). Secondary evaluation was performed to clarify the predictors of OTSC success.

RESULTS

The TSR, CSR, complication rate, and median procedure time were 89.7%, 84.5%, 1.8%, and 8 (range 1-36) min, respectively, demonstrating good outcomes. However, significant differences were observed between the two groups in terms of the mean procedure time (5.9 min *vs* 14.1 min). The CSR of the SS- and TG-groups among cases with a maximum defect size $\leqslant$ 10 mm and immediate or acute refractory bleeding was 100%, which suggests that SS is a better method than TG in terms of time efficacy. The CSR in the SS-group (78.6%), despite the technical success of the SS method (TSR, 100%), tended to decrease due to delayed leakage compared to that in the TG-group (TSR, CSR; 88.1%), indicating that TG may be desirable for leaks and fistulae with defects of the entire layer.

CONCLUSION

OTSC system is a safe and effective therapeutic option for gastrointestinal defects. Individualized selection of the suction method based on particular clinical conditions may contribute to the improvement of OTSC success.

Key words: Over-the-scope clip; Leak; Gastrointestinal refractory bleeding; Fistula; Endoscopic closure

Core tip: The efficacy of over-the-scope clips (OTSCs) for gastrointestinal defects has been widely known. However, few large studies with more than 50 cases have been performed. Additionally, an optimal strategy for selecting a suction method, which is a critical factor of OTSC success, is needed. This study, with a large number of cases and a multicenter design, demonstrated excellent outcomes of OTSC and revealed which type of suction method was appropriate for particular situations according to the following characteristics: defect size, duration since onset, and indication. The individualized choice of the suction method is the most important factor determining OTSC success.

Kobara H, Mori H, Fujihara S, Nishiyama N, Chiyo T, Yamada T, Fujiwara M, Okano K, Suzuki Y, Murota M, Ikeda Y, Oryu M, AboEllail M, Masaki T. Outcomes of gastrointestinal defect closure with an over-the-scope clip system in a multicenter experience: An analysis of a successful suction method. *World J Gastroenterol* 2017; 23(9): 1645-1656 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1645.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1645

INTRODUCTION

Conventional endoscopic therapy-resistant gastrointestina diseases have traditionally required invasive surgery. These diseases mainly consist of GI refractory bleeding and leaks, including perforations, anastomotic leakage, and fistulae, which are encountered during endoscopic evaluation and are related to significant morbidity and mortality[1]. Recently, with the development of endoscopic submucosal dissection (ESD)[2] and natural orifice transluminal endoscopic surgery (NOTES)[3], technological advances in endoscopic devices have allowed for the endoscopic closure of GI defects. Among several full-thickness suturing devices[4,5], the over-the-scope clip (OTSC) (Ovesco Endoscopy GmbH, Tübingen, Germany) has the advantage of rapid and convenient use in rescue therapy. Currently, many case reports[6-10] and preliminary case series[11-14] have reported on the efficacy of OTSCs for the closure of GI defects, eliminating the need for invasive surgery.

However, there are few studies that have used large samples[15], and few randomized controlled trials[16,17] have been performed with OTSCs. Specifically, a strategy for choosing a suction method into the application cap of the OTSC system has not been clearly described. Successful OTSC closure depends on the secure suction of the target lesion into the application cap. The options available for OTSC closure include three suction methods, including simple suction (SS), which is similar to endoscopic variceal band ligation, and two accessory devices (Ovesco Endoscopy GmbH), which are referred to as the Twin Grasper (TG) and the tissue-anchoring device called the Anchor. Functioning as grasping forceps, the TG is applied to easily approximate the grasping edges of a large lesion, whereas the Anchor can better approximate indurated tissue. As both devices are expensive, selection of the appropriate suction method needs to be made according to the characteristics of the target lesion, which include the size of the defect, indications, and the duration since onset. The primary goal

Table 1 Demographics and characteristics of patients, defects, and over-the-scope clips

Characteristics	Details	Total patients (n = 58)
Age, median (range), yr		77 (37-98)
Indications, *n*		
Refractory bleeding		18
	Ulcer (peptic, Behçet's, anastomosis)	12
	Mallory-Weiss tear	1
	Diverticula	2
	Post-endoscopic resection	3
Leaks		28
	Peptic ulcer	3
	Boerhaave	1
	Iatrogenic (ESD)	16
	Iatrogenic (ERCP)	2
	Iatrogenic (surgery)	4
	Iatrogenic (other)	2
Fistula		12
	PEG	6
	Rectum-bladder	1
	Rectum-pelvis	2
	Gastric tube-trachea	1
	Gastric-pseudopancreatic cyst	1
	Colon-gallbladder	1
Location, *n*		
Esophagus		3
Stomach		28
Duodenum		13
Small intestine		2
Colon		12
Maximum defect size (D) mm, *n*		
D ⩽ 10		25
10 < D ⩽ 20		9
20 < D		24
Median (range), mm		15 (3-50)
Duration since onset to OTSC placement, *n*		
Immediate ⩽ 1 d		25
1 < Acute ⩽ 7 d		11
Chronic > 7 d		22
Suction method into the applicator cap		
Simple suction		14
Twin Grasper (TG) assist		42
Anchor assist		2
The number of OTSC deployments, *n*		
0		2[1]
1		39
2		12
3		5

[1]Procedural inability. OTSC: Over-the-scope clip; PEG: Percutaneous endoscopic gastrostomy; ERCP: Endoscopic retrograde cholangiopancreatography; SS: Simple suction; TG: Twin Grasper.

of this study was to demonstrate clinical outcomes of a multicenter experience with OTSCs for the management of GI refractory bleeding, leaks, and fistulae. The secondary goals were to propose a directional strategy for choosing a suction method into the application cap of the OTSC system by comparing the clinical data of SS to that of TG.

MATERIALS AND METHODS

Study design

This retrospective study was conducted at 5 medical centers in the Shikoku area of Japan. Between November 2011 and November 2015, fifty-eight patients who underwent attempted OTSC placement for GI refractory bleeding, leaks, or fistulae were enrolled. The detailed clinical data are summarized in Table 1. Patient characteristics, including age, indications with details, location of the defect, maximum defect size (D, mm), duration from onset to OTSC placement (immediate, ⩽ 1 d, acute, 1-7 d, or chronic, > 7 d), and the numbers of OTSC deployments, were collected. The indication for OTSC application for GI nonvariceal and refractory bleeding was defined as cases in which 2 time trials by conventional interventions failed to achieve complete hemostasis. Perforations, deep defects of the gut with the risk of delayed perforations, and anastomotic leakages were included as leaks. Subsequently, 56 patients, excluding two cases that used the Anchor, were divided into two groups: 14 cases of simple suction (SS-group) *vs* 42 cases using the Twin Grasper (TG-group). All of the data were extracted and compiled into a central database at Kagawa University. Written informed consents related to the use of OTSCs were obtained from all patients. The Clinical Ethics Committee of Kagawa University Hospital and each institution approved this study. This study was registered under UMIN 000017767.

OTSC procedures

The OTSC system is primarily composed of an OTSC mounted onto an application cap and a hand wheel. Users can easily apply the simple mechanism. As previously reported[18], the OTSC procedure involved several steps. First, the endoscope on which the cap with the loaded OTSC was mounted was inserted into the GI tract either orally or anally. Either a gastroscope (GIF-Q260J, ø 9.9 mm or H260Z, ø 10.8 mm Olympus, Tokyo, Japan) or a colonoscope (PCF-Q260AI, ø 11.3 mm, Olympus) with a maximum diameter of 9.9 mm and a working channel with greater than a 2.8 mm diameter was applied. Second, the defect in the GI tract was sucked to an application cap using SS or application aids such as the TG or the Anchor. The choice of the suction method ultimately depended on the discretion of the operator in this study. Finally, the clip was fired by stretching the wire with the hand wheel, and the entire defect of the lesion was completely closed. The OTSC procedures for the SS and TG methods and the Anchor assist are shown as schemas in Figure 1. Additional OTSCs were deployed until the defect was entirely closed. Regarding the types of OTSCs that were used, the gastrostomy closure type for gastric walls and the traumatic (t) type for other organs with thin walls were introduced, depending

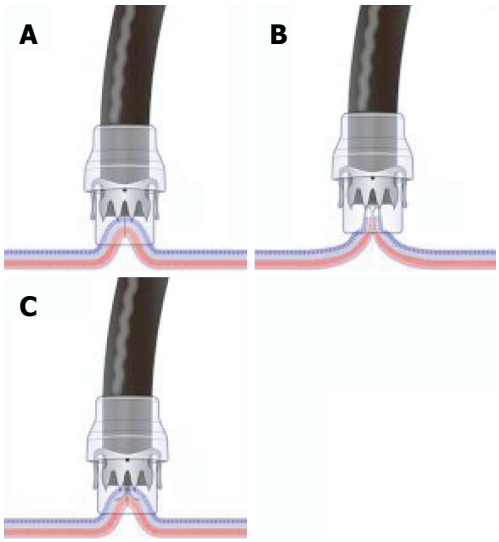

Figure 1 Key factor for the success of over-the-scope clips: schema of three suction methods into the application cap of the target lesion. A: Simple suction, similar to endoscopic variceal band ligation (simple suction method); B: Assist of grasping forceps: Twin Grasper device (Twin Grasper method); C: Assist of tissue anchoring device (Anchor assist).

on the lesion and the assessment of the operator; the atraumatic (a) type was not used in this study. Six expert endoscopists (H.K., H.M., T.Y., N.N., M.M., and M.O.) who had gained experience with the OTSC system procedure in a porcine model during a hands-on seminar session performed the OTSC deployments.

Outcome measures

Major outcomes: The overall rates of technical success (TSR), clinical success (CSR), complications, and procedure time of the 58 patients were examined. Technical success was defined as the complete closure of the entire defect by the successful deployment of OTSCs. Clinical success was defined as the resolution of the troubled situation by the assessment of blood analysis, endoscopic, and/or radiographic imaging (surgery or further endoscopic intervention was not required during at least 1 mo of follow-up after OTSC placement). The procedure time of the suction method was defined as the duration between the attempts at aspiration or the application of the TG or Anchor on the target lesion and complete closure of the defect with OTSC placement, as reviewed by endoscopic images and/or movies. The number of OTSC placements per single defect was calculated when the entire defect of the lesion was completely closed.

Secondary outcomes: A secondary evaluation was performed to clarify the predictors of OTSC success in the SS- and TG-groups. The TSR, CSR, procedure time, and complication rates of both groups were compared.

Subsequently, the TSR and CSR of each parameter and the indications, location of the defect, maximum defect size (≤ 10, 10-20, or > 20 mm), and duration since onset (immediate, acute, or chronic) were compared between the SS- and TG-groups. We supposed that a maximum defect size of 10 mm might be suitable for complete closure in the SS-group, considering the caliber of the application cap (11 or 12 mm in diameter). Previous studies have shown that factors that promote OTSC failure include a large defect size (greater than 20 mm)[14], fibrosis of the target tissue, such as a fistula, and the duration from onset to OTSC placement[15]. Thus, the maximum defect size was defined using the cut-off values of 10 and 20 mm, and the duration from onset was evaluated as one parameter. Simultaneously, the CSRs in both groups in terms of the combined parameters, the defect size, and the duration since the onset of each indication were estimated to better clarify the quality of each method.

Table 2 Results of major outcomes

Outcomes	Total patients (n = 58)
Technical success rate, % (95%CI)	89.7 (81.0-98.4)
Clinical success rate, % (95%CI)	84.5 (74.3-94.7)
Complications, n (%)	1 (1.8) in 56 cases used
The procedure time, median (range), min	8 (1-36) in 52 successful cases

Statistical analysis

Normally distributed data are presented as medians and ranges. The TSRs, CSRs and complication rates in the SS- and TG-groups were compared using two-sided Fisher's exact tests. The mean procedure times of both methods were compared using two-sided Wilcoxon/Kruskal-Wallis tests. The TSRs and CSRs of each parameter were compared using a χ^2 test. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using JMP version 9.0 (SAS Institute Inc., Cary, NC, United States).

RESULTS

The results for the major outcomes are summarized in Table 2. The TSR and CSR were 89.7% and 84.5%, respectively. The complication rate was 1.8% in the 56 cases analyzed. The median procedure time (range) was 8 (1-36) min in 52 successful cases. Additionally, the TSR and CSR of each parameter are shown in Table 3. While the TSR decreased as defect size and duration since onset increased, the CSR decreased as duration since onset increased.

The results of the comparison between the SS- and TG-groups with respect to the major outcomes are summarized in Table 4. No significant differences were identified between the SS- and TG-groups in terms of TSR [100% (14/14) *vs* 88.1% (37/42), respectively] and CSR [78.6% (11/14) *vs* 88.1% (37/42), respectively], $P > 0.05$). However, the CSR in the SS-group [78.6% (11/14)], despite the technical

Table 3 Results of the technical and clinical success rates for each parameter

Parameters	Total patients (n = 58)	
	Technical success rate	Clinical success rate
Indications		
Refractory bleeding	88.9 (16/18)	83.3 (15/18)
Leak	89.3 (25/28)	85.7 (24/28)
Fistula	91.7 (11/12)	83.3 (10/12)
Location		
Upper GI tract	86.4(38/44)	81.8 (36/44)
Lower GI tract	100 (14/14)	92.9 (13/14)
Maximum defect size (D), mm		
D ⩽ 10	96 (24/25)	84 (21/25)
10 < D ⩽ 20	88.9 (8/9)	88.9 (8/9)
20 < D	83.3 (20/24)	83.3 (20/24)
Duration since onset, % (n)		
Immediate ⩽ 1 d	96 (24/25)	96 (24/25)
1 < Acute ⩽ 7 d	90.9 (10/11)	81.8 (9/11)
Chronic > 7 d	81.8 (18/22)	72.7 (16/22)
Suction method into the applicator cap		
Simple suction (SS)	100 (14/14)	78.6 (11/14)
Twin Grasper (TG)	88.1 (37/42)	88.1 (37/42)
Anchor assist	50 (1/2)	50 (1/2)
The number of OTSC deployments, n		
1	92.3 (36/39)	84.6 (33/39)
2	100 (12/12)	100 (12/12)
3	80 (4/5)	80 (4/5)

success of the procedure (TSR, 100%), tended to decrease compared to that in the TG-group (TSR, CSR; 88.1%). Additionally, no significant differences were identified between the two groups in terms of the rate of complications [0% (0/14) *vs* 2.4% (1/42), $P > 0.05$]. However, significant differences were observed between the two groups regarding the mean procedure time (SS, 5.9 *vs* TG, 14.1 min, $P < 0.05$). A flow diagram of patient enrollment and outcomes is illustrated in Figure 2.

There were no significant differences in the TSRs and CSRs between the two groups for any parameter ($P > 0.05$) (Table 5). The CSR in the TG-group decreased as defect size and duration since onset increased. The CSRs of the combined parameters, defect sizes and duration since onset in each indication are summarized in Table 6. For refractory bleeding, the CSRs for cases of D ⩽ 10 were 85.7% (6/7) in the SS-group and 100% (2/2) in the TG-group. The CSR of the SS- and TG-groups among cases with D ⩽ 10 and immediate or acute refractory bleeding was 100%, which suggested that SS is a better method than TG in terms of time efficacy. However, the CSRs of cases with leaks and fistulae and D ⩽ 10 were 71.4% (5/7) in the SS-group and 100% (7/7) in the TG-group. Delayed leakages occurred in two cases in the SS-group (D ⩽ 10 and acute leakage and D ⩽ 10 and chronic fistula). These data suggest that the SS method sometimes fails to provide an acceptable clinical outcome despite the technical success, even if the defect size is small (D

Table 4 Outcomes of the simple suction and Twin Grasper methods n (%)

Parameters	SS-method (n = 14)	TG-method (n = 42)	P value
Technical success rate	14 (100)	37 (88.1)	0.176[1]
Clinical success rate	11 (78.6)	37 (88.1)	0.378[1]
Procedure time, median (range), min	5 (1-16)	12 (3-36) in 37 successful cases	0.0004[2]
Complications	0 (0)	1 (2.4)	0.587[1]

[1]Fisher's exact test (2-sided), [2]Wilcoxon/Kruskal-Wallis test. SS: Simple suction; TG: Twin Grasper.

⩽ 10). These aspects of the SS method suggest that the TG is desirable for leaks and fistulae with defects of the entire layer.

Case presentation

A representative success of SS in refractory bleeding is shown in Figure 3. In a failure case in the SS-group with D ⩽ 10 and a chronic duration, conventional therapy-resistant ulcer bleeding that in the terminal ileum occurred during steroid treatment for myelodysplastic syndrome. Despite the successful closure of the defect with the SS method, additional surgery was needed because of re-bleeding that might have been caused by angiogenesis from the steroid treatment in specific circumstances (Table 7, case No. 1). The CSR of the TG-group among cases with D > 20 showed the lowest success rate, 33.3% (1/3). In these 2 failure cases of chronic, fibrotic ulcers with D > 20, technical success could not be achieved due to an inability to suck rigid tissues into the cap, even when using the TG (Table 7, case No. 2 and No. 3). Although the use of the Anchor might have been helpful[18], we did not introduce the device because of the risk of perforation by the bear claw of the device. Finally, these bleeds were managed with conventional therapies using hemostatic forceps. At the indication of a leak, the TG method provided good clinical outcomes for defects with D ⩽ 10 and immediate duration during endoscopic retrograde cholangiopancreatography (ERCP); an image of a representative case is shown in Figure 4. On the other hand, there were three clinical failure cases with leaks: one with SS with D ⩽ 10 and an acute duration, one with the TG with 10 < D ⩽ 20 and an immediate duration, and one with the TG with D > 20 and an acute duration. In the first case, an acute anastomotic leakage with D ⩽ 10 after surgery for gastric cancer that was located in the esophageal-gastric junction was successfully closed using SS, but additional surgery was needed because of a delayed leakage (Table 7, case No. 4). In the second case, a large perforation of approximately 20 mm occurred during ERCP, and the TG was used to approximate the defect. The collapse of the intestine because of air leakage made technical success impossible, and this

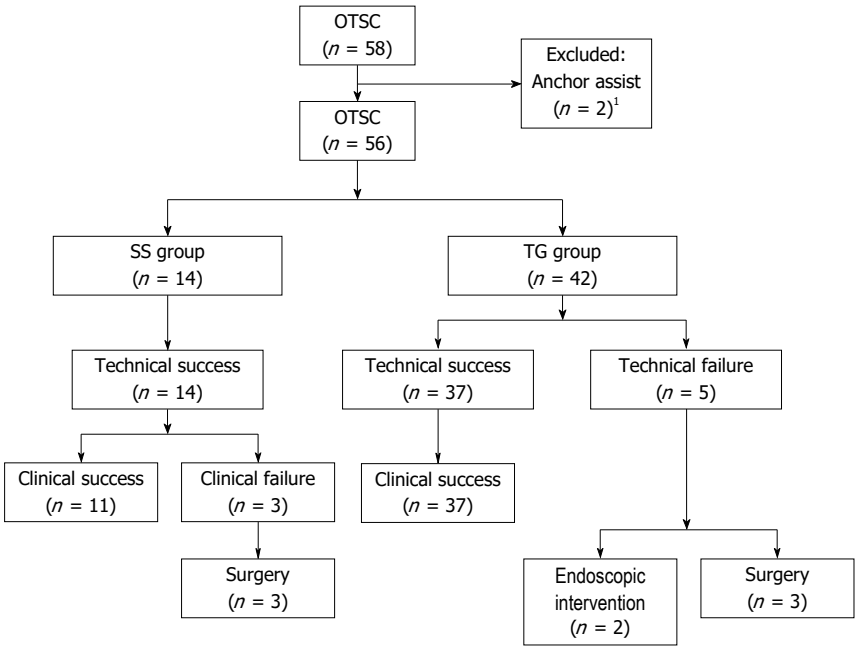

Figure 2 Flow diagram of patient enrollment and outcomes. [1]Clinical outcomes of two cases that used the Anchor: one case with an incomplete closure of an esophageal-gastric anastomotic leakage, and another case with clinical success of gastric fistula after percutaneous endoscopic gastrostomy. SS: Simple suction; TG: Twin Grasper.

Table 5 Results of the technical and clinical success rates of the simple suction- and Twin Grasper-groups for each parameter

Parameters	Technical success rate			Clinical success rate		
	SS (n = 14)	TG (n = 42)	P value[1]	SS (n = 14)	TG (n = 42)	P value[1]
Indication						
Refractory bleeding	100 (7/7)	81.8 (9/11)	0.2315	85.7 (6/7)	81.8 (9/11)	0.8288
Leak	100 (2/2)	92 (23/25)	0.6776	50 (1/2)	92 (23/25)	0.0690
Fistula	100 (5/5)	83.3 (5/6)	0.3384	80 (4/5)	83.3 (5/6)	0.8865
Location						
Upper GI tract	100 (8/8)	85.3 (29/34)	0.8725	75 (6/8)	85.3 (29/34)	0.8725
Lower GI tract	100 (6/6)	100 (8/8)		83.3 (5/6)	100 (8/8)	0.2308
Maximum defect size (D), mm						
D ⩽ 10	100 (14/14)	100 (9/9)		78.6 (11/14)	100 (9/9)	0.1364
10 < D ⩽ 20	-	88.9 (8/9)	-	-	88.9 (8/9)	-
20 < D	-	83.3 (20/24)	-	-	83.3 (20/24)	-
Duration since onset, % (n)						
Immediate ⩽ 1 d	100 (3/3)	95.5 (21/22)	0.6994	100 (3/3)	95.5 (21/22)	0.6994
1 < Acute ⩽ 7 d	100 (3/3)	87.5 (7/8)	0.1247	66.7 (2/3)	87.5 (7/8)	0.1247
Chronic > 7 d	100 (8/8)	75 (9/12)	0.1250	75 (6/8)	75 (9/12)	1.0000

[1]χ^2 test (2-sided). SS: Simple suction; TG: Twin Grasper.

case required surgical repair (Table 7, case No. 5). In the third case, a delayed perforation with a 50-mm defect size occurred after gastric endoscopic submucosal dissection. The defect could not be closed with the TG because of a narrow lumen in the prepylorus and the large defect size. The misplacement of the OTSC on the exposed muscularis propria induced additional tears, which represents the only case of an OTSC complication in this study. Although the use of several hemoclips at the perforation site seemed to be effective, surgery was performed due to the re-appearance of free air in computed tomography (CT) images 3 d after endoscopic therapy (Table 7, case No. 6, shown as the only complication in Figure 5). Among the fistula cases, there were two clinical failures: one with SS with D ⩽ 10 and a chronic duration and one with TG with 10 < D ⩽ 20 and a chronic duration. The first case was an 8-mm gastric fistula that occurred after an interventional endoscopic ultrasound for a pseudopancreatic cyst (Table 7, case No. 7), which is shown in Figure 6. Although the fistula was successfully closed using SS, leakage occurred 2 wk after OTSC placement, which necessitated additional surgery. The other case was a large (22 mm in diameter) gastric tube-tracheal fistula that occurred after radiation for esophageal carcinoma. The fistula could not be treated with the TG and required surgery (Table 7, case No. 8). Details of these 8 clinical failure cases are summarized

Table 6 Comparison of the clinical success rates for the combined parameters in each indication

Indications	Clinical success rate					
	Refractory bleeding (n = 18)		Leak (n = 27)		Fistula (n = 11)	
Combined parameters	SS (n = 7)	TG (n = 11)	SS (n = 2)	TG (n = 25)	SS (n = 5)	TG (n = 6)
D ≤ 10, Immediate	100%	100%	-	100%	-	-
D ≤ 10, Acute	100%	-	50%	100%	-	-
D ≤ 10, Chronic	66.7%	100%	-	-	80%	100%
10 < D ≤ 20, Immediate	-	100%	-	66.7%	-	-
10 < D ≤ 20, Acute	-	100%	-	100%	-	-
10 < D ≤ 20, Chronic	-	100%	-	-	-	100%
D > 20, Immediate	-	100%	-	100%	-	-
D > 20, Acute	-	-	-	75%	-	-
D > 20, Chronic	-	33.3%	-	-	-	50%

SS: Simple suction; TG: Twin Grasper.

Table 7 Details of over-the-scope clips clinical failure cases (n = 8)

Indication	Max. defect size, mm	Cause, comorbidity	Location	Prior therapy	Duration since onset	Technical success	Technical or clinical failure factor	Additional therapy	Clinical outcome
Refractory bleeding	8	Ileal ulcer bleeding due to steroid treatment for myelodysplastic syndrome	Terminal ileum	EI (hemoclips and coagulation)	Chronic	Yes	Suspicion of angiogenesis due to steroid in particular circumstances	Elective surgery	Survival
Refractory bleeding	20	Peptic ulcer, Refractory neurogenic disease	Stomach (body)	EI (coagulation)	Chronic	No	Fibrotic tissue	Retry of EI	Survival
Refractory bleeding	50	Peptic ulcer, Advanced gallbladder carcinoma	Stomach (body)	EI (coagulation)	Chronic	No	Fibrotic tissue	Retry of EI	Survival
Leak	7	Anastomotic leakage after surgery for gastric cancer	Esophageal gastric junction	None	Acute	Yes	Leakage by mucosal suture (suspected)	Elective surgery	Survival
Leak	21	Perforation during ERCP	Duodenal 2nd portion	None	Immediate	No	Inability of platform	Emergency surgery	Survival
Leak	50	Delayed perforation after ESD	Stomach (prepyrolus)	None	Acute	No	Location with narrow lumen	Elective surgery	Survival
Fistula	8	Interventional EUS	Gastric (prepyrolus)-pseudopancreatic cyst	None	Chronic	Yes	Leakage by mucosal suture (suspected)	Elective surgery	Survival
Fistula	22	Radiation for esophageal carcinoma	Gastric tube-trachea	Bronchial embolization	Chronic	No	Fibrotic tissue	Elective surgery	Survival

SS: Simple suction; TG: Twin Grasper; EI: Endoscopic intervention; ERCP: Endoscopic retrograde cholangiopancreatography; EUS: Endoscopic ultrasound.

in Table 7.

DISCUSSION

A newly developed endoscopic full-thickness suturing device, the OTSC system, has allowed for the endoscopic closure of conventional therapy-resistant GI defects. The efficacy of OTSC has been widely known since its introduction in 2009 in Western countries. However, there have been few studies that used large samples of more than fifty cases and a multicenter design.

Additionally, the type of suction method that should be applied to each target lesion based on the particular lesion characteristics remains unclear. Successful OTSC closure depends on the secure suction of the target lesion into the application cap. This success is closely related to the extent of tissue fibrosis in proportion to the duration from onset to OTSC placement, as previously described[14,15]. Therefore, an optimal strategy for choosing a suction method for the OTSC system is needed. This study is the first to clarify these issues by comparing the clinical data of SS to TG.

SS vs TG

Compared to TG, SS has the advantage of rapid and convenient use with a system that is similar to endoscopic variceal band ligation (mean procedure time; SS 5.9 min *vs* TG 14.1 min, $P < 0.05$). Moreover, another merit of SS is its lower cost if accessory devices are not applied. A maximum defect size of 10 mm per clip can be completely closed with the SS method, considering the caliber of the application cap. If OTSC is not fired due to insufficient suction into the cap during SS method, TG assist can be an alternative choice to close the defect. Although no significant differences in TSR or CSR were observed between the two groups in this study, the CSR of the SS-group (78.6%, 11/14),

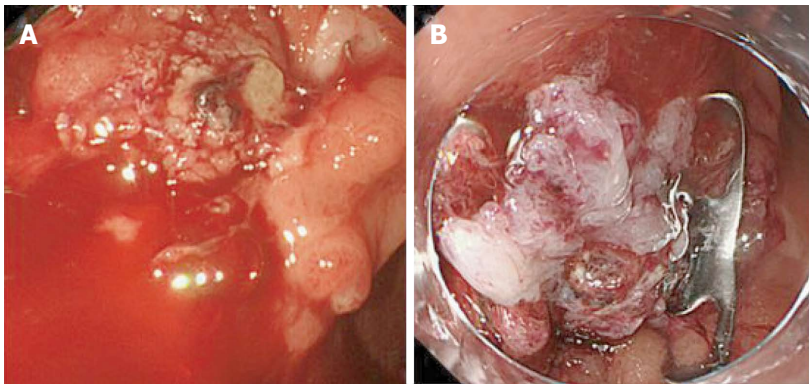

Figure 3 Representative clinical success case in the simple suction-group that exhibited refractory bleeding with a defect size of ⩽ 10 mm and an immediate duration since onset. A: A spurting, bleeding ulcer that was located in a rectal anastomotic site; B: Complete hemostasis *via* over-the-scope clip closure using the simple suction method after the failure of conventional endoscopic intervention.

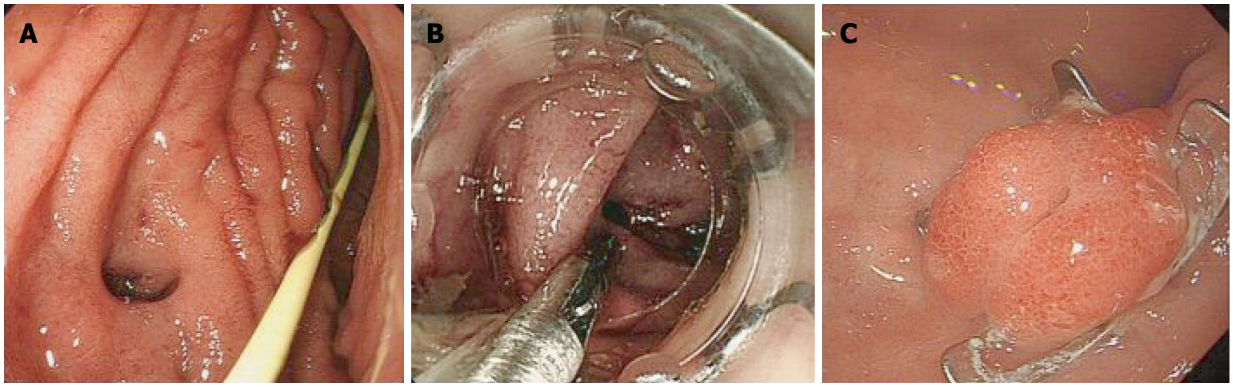

Figure 4 Representative clinical success case in the Twin Grasper-group of a leak with a defect size of ⩽ 10 mm and an immediate duration since onset. A: An iatrogenic perforation site approximately 10 mm in size, located in the 2nd portion of the duodenum during endoscopic retrograde cholangiopancreatography; B: Application of the Twin Grasper device; C: Complete defect closure three months after over-the-scope clip deployment.

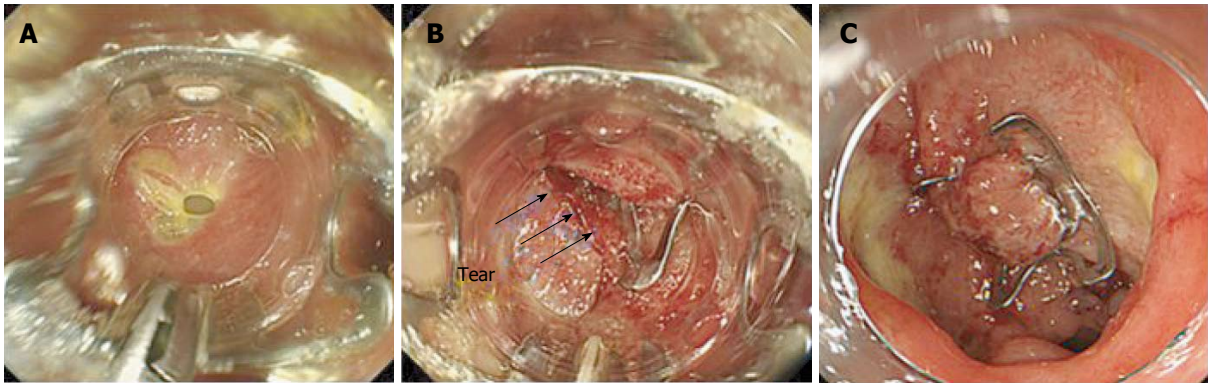

Figure 5 Representative case with an over-the-scope clips complication. A: A delayed perforation with a 50-mm defect size after gastric endoscopic submucosal dissection; B: The misplacement of the over-the-scope clips to the exposed muscularis propria induced additional tears (black arrows); C: The defect could not be closed by the Twin Grasper due to a narrow lumen located in the prepylorus.

despite its technical success (TSR, 100%, 14/14), tended to decrease compared to the TG-group (TSR, CSR; 88.1%, 37/42) due to delayed leakage. This finding indicates that the SS method might result in some clinical failures despite its technical success. Therefore, a secondary evaluation regarding each parameter or combined parameters for each indication was performed to better clarify the quality of each method. The CSR of the SS-group with D ⩽ 10 and immediate or acute refractory bleeding was 100%, which suggested that SS was a better method than TG in terms of time efficacy. However, the CSRs of leaks and fistulae with D ⩽ 10 were 71.4% (5/7) in the SS-group and 100% (7/7) in the TG-group. Delayed

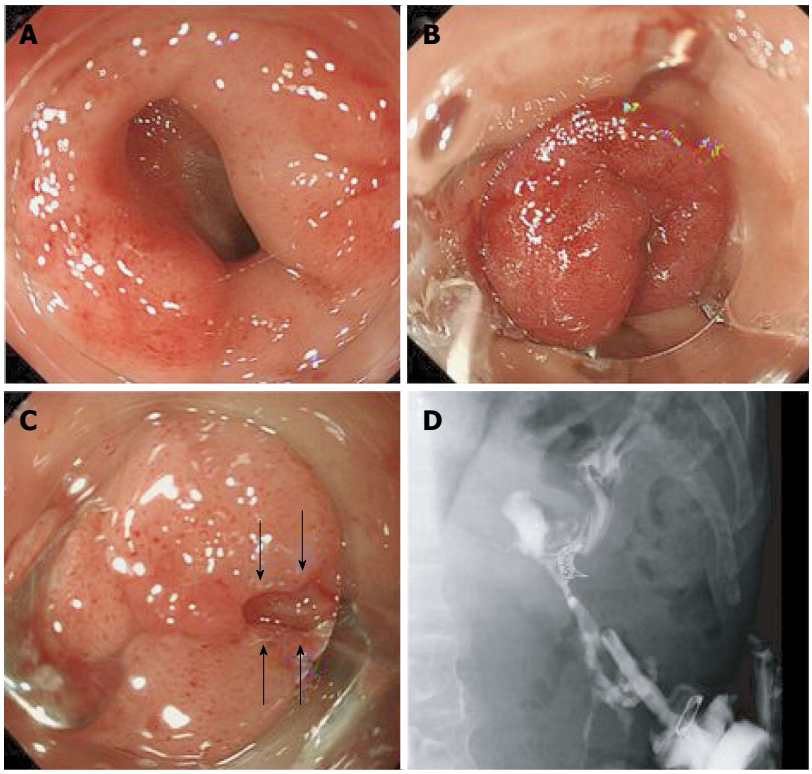

Figure 6 Clinically unsuccessful case in the simple suction-group of a fistula with a defect size of ⩽ 10 mm and a chronic duration. A: An 8-mm gastric fistula after interventional endoscopic ultrasound for a pseudo-pancreatic cyst; B: Complete closure with one over-the-scope clip (OTSC) using the simple suction method; C: A delayed leakage (black arrows) that occurred 2 wk after OTSC placement; D: X-ray contrast photo via a percutaneous drainage tube that revealed the delayed leakage.

leakages occurred in two cases in the SS-group (D ⩽ 10 and acute leakage and D ⩽ 10 and chronic fistula). These interesting data suggest that the SS method might sometimes fail in full-thickness suturing and could result in mucosal suturing for the target tissue, even if the defect size is small. Moreover, OTSC system using TG assist after clinical failure of SS method is not applicable for the same defect, because it is difficult to remove endoscopically the deployed OTSC on the target lesion. In this situation, surgery will be the only suitable therapy as shown in Figure 2. Thus, the use of TG may be desirable for leaks and fistulae with defects of the entire layer.

The TG is commonly used for large defects, but details regarding defect sizes and indications are unknown. The CSR in the TG-group decreased as defect size and duration since onset increased (Table 5). In particular, the success rate in the TG-group was the lowest for defects with D > 20 and those that were chronic, which indicates the limitations of the TG for large defects with fibrosis. If the TG method fails in this condition, a retrial with the Anchor might be valuable. Further comparative studies that include the Anchor are needed to clarify its efficacy and limitations.

Overall clinical success

Currently, there are limited data from large sample sizes[15] and few comparative studies[16,17]. Specifically, clinical studies including more than 50 cases, as in the present study, are rare. Here, we summarized the overall CSR in human studies between 2011 and 2015 that involved a minimum of 2 wk of follow-up, and we included several important parameters (*e.g.,* defect size, use of accessory devices) (Table 8)[19-26]. The mean rate of overall clinical success was 68.4% (range 53-90) (329/481 cases), which includes our results. Our data revealed a high rate of overall clinical success (84.5%). This finding may be why no significant differences in TSR or CSR were observed between the two groups in this study. Additionally, the proportion of fistulae and/or the defect size included in other studies may be associated with the OTSC success rate. According to a large sample of data, a defect type with fibrotic tissue, such as a fistula, is the most important predictor of OTSC failure[15]. Therefore, we evaluated the success rates of three types of indications separately (refractory bleeding, leaks, and fistulae). The mean rates of overall clinical success in refractory bleeding, leaks, and fistulae were 87.8%, (79/90 cases), 83.2% (109/131), and 53.0% (133/251), respectively.

Similar to the overall mean rate of complications (1.66%, 8/481), there was only one case in which the misplacement of the OTSC to exposed muscularis propria induced additional tears in this study (1.8% complication rate). Although OTSCs have been dem-

Table 8 Current clinical outcomes of over-the-scope clips for each indication

Ref.	Year	Country	Patients (*n*)	Overall clinical success rate, (success/ total)					Mean defect size (mm)	Described data of suction method	Complications, *n* (%)
				Refractory bleeding	Leaks and/or perforations	Fistula	Others	Total			
Albert *et al*[20]	2011	Germany	19	57.1 (4/7)	87.5 (7/8)	25 (1/4)	-	63.2	Unknown	Unknown	0
Surace *et al*[21]	2011	France, Monaco	19	-	-	74 (14/19)	-	73.7	Unknown	Unknown	1 (5)
Kirschniak *et al*[22]	2011	Germany	50	92.6 (25/27)	100 (11/11)	37.5 (3/8)	100 (4/4)	86	6	Unknown	0
Baron *et al*[19]	2012	United States	45	100 (7/7)	62.5 (5/8)	67.9 (19/28)	50 (1/2)	71	Unknown	TG: 8 cases AC: 17 cases	2 (4.4)
Manta *et al*[23]	2013	Italy	30	90 (27/30)	-	-	-	90	Unknown	Unknown	0
Haito-Chavez *et al*[15]	2014	International[2]	188	-	Leaks 73.3 (22/30), 2[1] Perforations 90 (36/40), 8[1]	42.9 (39/91), 17[1]	-	60.2	Leaks: 8 Perforations: 7 Fistula: 5	Use of TG and/or AC, 50% (70/140 cases)[3]	0
Law *et al*[24]	2014	United States	47	-	-	53 (25/47)	-	53	Unknown	Unknown	0
Sulz *et al*[25]	2014	Switzerland	21	100 (1/1)	66.7 (4/6)	63.6 (7/11)	100 (3/3)	71.4	8	SS:[4] 100% (10/10) TG:100% (1/1) AC: 87.5% (7/8) TG + AC: 0% (0/1)	0
Mercky *et al*[26]	2015	France, Monaco	30	-	-	53 (16/30)	-	53	7.2	SS: 17 procedures TG: 9 procedures AC: 5 procedures	4 (13.3)
Our study	2016	Japan	58	83.3 (15/18)	85.7 (24/28)	83.3 (10/12)	-	84.5	19.6	CSR SS[4]:78.6% (11/14) TG: 88.1% (37/42) AC: 50% (1/2)	1 (1.8)
Total			507	87.8 (79/90)	83.2 (109/131)	53.0 (133/251)	88.9 (8/9)	68.4 (329/481)			8/481 (1.66)

[1]Excluded number of patients lost to follow-up; [2]United States, Netherlands, Germany, Italy, and Chile; [3]Use of accessory devices was not a predictor of clinical success; [4]Clinical success rate. SS: Simple suction; TG: Twin Grasper; AC: Anchor.

onstrated to be safe, a careful approach is needed to avoid OTSC placement on exposed muscularis propria, which can occur in a defect after endoscopic resection.

GI refractory bleeding

The OTSC system offers the strongest impact in regards to GI bleeding compared to other indications, as evidenced by the mean rate of overall clinical success of 87.8%, (79/90 cases), which is similar to the findings in our study (83.3%, 15/18). Therefore, an OTSC is a good device with which to achieve hemostasis in conventional therapy-resistant GI bleeding. However, as our 2 failure cases with D > 20 and chronic fibrotic ulcers revealed, OTSC usage may be limited in particular situations.

Leaks

Perforations, deep defects with the risk of delayed perforations, and anastomotic leakages were included as leaks in this study. As the mean rate of overall clinical success and our CSR were 83.2% (range 62.5-100) (109/131) and 85.7% (24/28), respectively, the OTSC is valuable in avoiding emergency surgery for leaks.

Fistulae

Among all of the indications, the mean rate of overall clinical success for fistula was the lowest (53.0%, range 25-81.8, 133/251 cases). Similarly, recent studies have demonstrated a limited success rate of approximately 50%. Despite the introduction of the OTSC system, fistula closure appears to be a challenge. However, compared to other studies, our study delivered good outcomes with both technical (91.7%, 11/12 cases) and clinical success (83.3%, 10/12 cases). Law *et al*[24] reported that nearly 50% of patients with endoscopic and radiologic evidence of fistula closure at completion of the index procedure went on to require additional interventions in the subsequent days and months due to fistula recurrence. Accordingly, we recommend the use of sufficient suc-

tion into the cap with the aggressive use of accessory devices for the successful long-term closure of fistulae. In the future, the issue of managing refractory fistulae may be overcome by utilizing one or more of the following modalities: the injection of tissue sealants[27], stent placement[28,29], and newly developed endoscopic suturing devices[4,5,30].

Limitations

The main limitation of this study is its retrospective design. Additionally, the selection of the suction method depended on the operator's discretion, so patient inclusion criteria were subjective. Therefore, the Anchor device was applicable only for a small number of cases in our experience.

Strengths of this study

This study has several strengths. Compared to related studies, it is a relatively large, multicenter study. Additionally, this study is the first to investigate which type of suction method is appropriate for particular situations according to the following characteristics: defect size, duration since onset, and indication.

In conclusion, the OTSC system is a safe and effective therapeutic option for the treatment of GI defects. The individualized choice of the suction method in the OTSC system is the most important factor for OTSC success. Thus, OTSCs can serve as reliable and productive devices for GI refractory diseases when the size of the defect, the duration since onset and the indication are considered.

COMMENTS

Background

Although the efficacy of over-the-scope clip (OTSC) for gastrointestinal (GI) defects involving GI refractory bleeding, leakages, and fistulae had been described, there are few data using large samples over fifty cases. Additionally, a successful key of OTSC closure depends on the secure suction into the application cap of the target lesion. There are three suction methods: simple suction (SS) and two accessory devices, referred to as the Twin Grasper (TG) and the tissue anchoring device, the Anchor. However, an optimal strategy for selecting a suction method, which is a critical factor of OTSC success, remains unclear.

Research frontiers

This study demonstrates clinical outcomes of OTSCs using large samples and proposes a directional strategy for choosing a suction method into the application cap of the OTSC system.

Innovations and breakthroughs

Compared to related studies, this is a multicenter study with a large number of cases. Additionally, this study is the first to investigate which type of suction method is appropriate for particular situations according to the following characteristics: defect size, duration since onset, and indication. Although the SS method is indicated for cases with a maximum defect size $\leqslant$ 10 mm and immediate or acute refractory bleeding in terms of time efficacy, SS sometimes fails in full-thickness suturing. Thus, the use of TG may be desirable for leaks and fistulae with defects of the entire layer.

Applications

This study emphasizes that OTSC system is a safe and effective therapeutic option for GI defects. Moreover, individualized selection of the suction method based on particular clinical conditions may contribute to the improvement of OTSC success.

Terminology

OTSC: A newly developed endoscopic full-thickness suturing device applicable for refractory bleeding, perforations, anastomotic leakage, and fistulae. Suction method: a method to suck the target lesion into the application cap, which is a critical factor of OTSC success among OTSC procedures.

Peer-review

The authors reported a multicenter retrospective study analyzing the role of the OTSCs for GI defects based on the suction method. The authors suggested that TG is desirable for leaks and fistulae with defects of the entire layer. However, further prospective studies by comparing suction methods are needed to clarify the type of suction method that should be applied to each target lesion based on the particular lesion characteristics.

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ORIGINAL ARTICLE

Observational Study

Usefulness of a novel slim type FlushKnife-BT over conventional FlushKnife-BT in esophageal endoscopic submucosal dissection

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Author contributions: Ohara Y, Toyonaga T and Hoshi N contributed mainly to this work; Tanaka S, Baba S, Takihara H, Kawara F and Ishida T participated in the analysis, and interpretation of the data; Morita Y, Umegaki E and Azuma T supervised the report.

Institutional review board statement: The study was reviewed and approved by the Ethics Committees of Kobe University Hospital No. 160051 and Kishiwada Tokushukai Hospital No. 28-10.

Informed consent statement: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest statement: Dr. Toyonaga invented FlushKnife-BT and FlushKnife-BTS in conjunction with Fujifilm and receives royalties from their sales.

Data sharing statement: No additional data are available.

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Abstract

AIM

To investigated the usefulness of a novel slim type ball-tipped FlushKnife (FlushKnife-BTS) over ball-tipped FlushKnife (FlushKnife-BT) in functional experiments and clinical practice.

METHODS

In order to evaluate the functionality of FlushKnife-BTS, water aspiration speed, resistance to knife insertion through the scope, and waterjet flushing speed were compared between FlushKnife-BTS and BT. In clinical practice, esophageal endoscopic submucosal dissection (ESD) performed using FlushKnife-BTS or BT by an experienced endoscopist between October 2015 and January 2016 were retrospectively reviewed. The treatment speed and frequency of removing and reinserting the knife to aspirate fluid and air during ESD sessions were analyzed.

RESULTS

Functional experiments revealed that water aspiration speed by the endoscope equipped with a 2.8-mm working channel with FlushKnife-BTS was 7.7-fold faster than that with conventional FlushKnife-BT. Resistance to knife insertion inside the scope with a 2.8-mm working channel was reduced by 40% with FlushKnife-BTS. The waterjet flushing speed was faster with the use of FlushKnife-BT. In clinical practice, a comparison of 6 and 7 ESD using FlushKnife-BT and BTS, respectively, revealed that the median treatment speed was 25.5 mm^2/min (range 19.6-30.3) in the BT group and 44.2 mm^2/min (range 15.5-55.4) in the BTS group (P = 0.0633). However, the median treatment speed was significantly faster with FlushKnife-BTS when the resection size was larger than 1000 m^2 (n = 4, median 24.2 mm^2/min, range 19.6-27.7 *vs* n = 4, median 47.4 mm^2/min, range 44.2-55.4, P = 0.0209). The frequency of knife replacement was less in the BTS group (median 1.76 times in one hour, range 0-5.45) than in the BT group (7.02 times in one hour, range 4.23-15) (P = 0.0065).

CONCLUSION

Our results indicate that FlushKnife-BTS enhances the performance of ESD, particularly for large lesions, by improving air and fluid aspiration and knife insertion during ESD and reducing the frequency of knife removal and reinsertion.

Key words: Endoscopic submucosal dissection; Novel slim type ball-tipped FlushKnife; ball-tipped FlushKnife; Resistance to knife insertion; Water aspiration speed

Core tip: Devices utilized in endoscopic submucosal dissection (ESD) play an important role in facilitating the safe and effective procedure. A novel slim type ball-tipped FlushKnife (FlushKnife-BTS) has been developed to enhance the performance of aspiration and insertion of the knife through the scope. We herein investigated the usefulness of FlushKnife-BTS over FlushKnife-BT in functional experiments and clinical practice. FlushKnife-BTS showed a faster water aspiration speed, reduced resistance to knife insertion, a faster treatment speed when the resection size was large, and low frequency of knife replacement. Our results indicate that FlushKnife-BTS supports the efficient performance of ESD, particularly for large lesions.

Ohara Y, Toyonaga T, Hoshi N, Tanaka S, Baba S, Takihara H, Kawara F, Ishida T, Morita Y, Umegaki E, Azuma T. Usefulness of a novel slim type FlushKnife-BT over conventional FlushKnife-BT in esophageal endoscopic submucosal dissection. *World J Gastroenterol* 2017; 23(9): 1657-1665 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1657.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1657

INTRODUCTION

Endoscopic submucosal dissection (ESD) is a standard treatment for early-stage tumors in the digestive tract[1-6]. Many devices have been developed to safely and efficiently facilitate this procedure[7-14]. One of these devices is FlushKnife and the subsequently developed ball-tipped FlushKnife (FlushKnife-BT) (DK2618JN, DK2618JB; Fujifilm Medical Co., Ltd., Tokyo, Japan)[13,14]. Some of the features and functions of these knives are advantageous such as injection, irrigation by waterjets, dissection, hemostasis, and vessel sealing[15]. However, when they are used with endoscopes equipped with a working channel of 2.8 mm, difficulties are associated with aspirating fluid and air in the working space and finely controlling the knife length due to limited space with friction resistance in the channel with a knife with a diameter of 2.7 mm.

Therefore, a novel slim type FlushKnife BT (FlushKnife-BTS, DK2620JBS; Fujifilm) was developed to provide more space in the working channel (Figure 1). FlushKnife-BTS is characterized by its slim sheath (2.2 mm), but same sized sheath tip (2.7 mm, approximately 30 mm long) as the conventional knife, which maintains stable maneuverability.

In an attempt to clarify whether the performance of ESD is better with FlushKnife-BTS than with FlushKnife-BT, we retrospectively investigated the usefulness of FlushKnife-BTS over FlushKnife-BT in functional experiments and clinical practice. In the clinical practice, we included esophageal ESD because esophagus is a narrow tract compared to stomach and colorectum, which procedure is affected easily by air inflation, and was thought to be a good candidate for first investigation into the efficiency of FlushKnife-BTS.

MATERIALS AND METHODS

Functional experiment

Water aspiration speed with the knife inserted in the scope, resistance to knife insertion inside the scope, and waterjet flushing speed were compared between FlushKnife-BTS and FlushKnife-BT. Regarding the speed of water absorption, a total of 200 mL water in a graduated cylinder was aspirated using the 2.8-mm scope and 3.2-mm channel with FlushKnife-BT or FlushKnife-BTS inserted, and the amount of water aspirated in 10 s was measured. The experiment was repeated 9 times. Resistance to the insertion of Flushknife-BT or Flushknife-BTS inside the scope was measured with various endoscopic angles. A measuring instrument named force gage FGP-5 produced by NIDEC-SHIMPO CORPORATION was equipped with the FlushKnife-BTS and FlushKnife-BT, inserted into the endoscope equipped with a working channel of 2.8 mm and the resistance during the insertion was determined. The experiment was repeated 3 times. The waterjet flushing speeds of the knives were measured

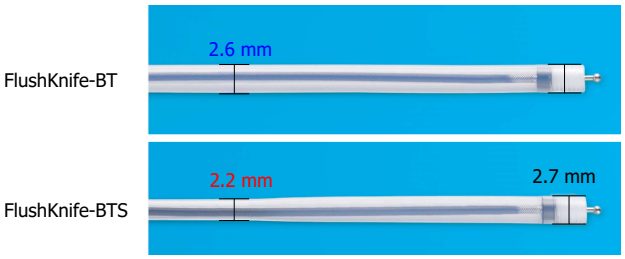

Figure 1 Ball-tipped FlushKnife and a novel slim type ball-tipped FlushKnife. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

at three different water pressure settings 9 times using waterjet equipment (JW-2; Fujifilm, waterjet volume; min/mid/max 80/135/190 mL/min).

Patients

All cases that underwent esophageal ESD performed using FlushKnife-BTS or FlushKnife-BT at Kobe University Hospital and Kishiwada Tokushukai Hospital between October 2015 and January 2016 were retrospectively reviewed. Of these, cases that underwent ESD performed by an experienced endoscopist (T.T.) were analyzed in the study.

Indications for esophageal ESD were defined according to the esophageal ESD guidelines issued by the Japan Esophageal Society[16]: (1) absolute criteria; lesions that do not exceed the mucosal layer (T1a), those remaining within the mucosal epithelium (EP) or the lamina propria mucosae (LPM), and (2) relative criteria; lesions reaching the muscularis mucosae (MM) or slightly infiltrating the submucosa (up to 200 μm, T1b-SM1). ESD was not recommended for a 10%-15% risk of lymph node metastasis with lesions filling the relative criteria; however, it was performed on patients who were too frail to tolerate more invasive surgical approaches due to comorbidities, those who requested a diagnostic endoscopic treatment before surgery, or those who requested ESD with chemoradiation.

All patients underwent an initial screening examination including esophagogastroduodenoscopy with magnified narrow band imaging and computed tomography in order to evaluate submucosal invasion and lymph node metastasis. When submucosal invasion was suspected, endoscopic ultrasonography was performed.

ESD procedure

Patients were mainly sedated using dexmedetomidine hydrochloride, flunitrazepam, and pentazocine. ESD was performed with a single-channel endoscope equipped with a working channel of 2.8 mm (GIF-Q240, H290Z; Olympus Corporation, Tokyo, Japan). ERBE VIO 300 D high performance cautery (Erbe Elektromedizin GmbH, Tubingen, Germany) was utilized in all cases. Carbon dioxide insufflation was routinely used.

The knife was removed and reinserted to aspirate fluid and air when required, to clean the endoscope lens for better visualization, and/or when the thread-traction method was needed[17].

Evaluated points

The patient and lesion characteristics including sex, age, lesion site, circumference of the resected area, major axis diameter of the resected specimen, size of resected area, major axis diameter of the tumor, histology of the tumor and depth of the tumor were investigated.

Furthermore, the procedure time, treatment speed, frequency of knife removal and reinsertion for aspiration, *en bloc* resection rate, and adverse events were assessed and compared between the FlushKnife-BTS (BTS) group and FlushKnife-BT (BT) group.

The procedure time was defined as the time between the first submucosal injection and completion of dissection. The treatment speed was calculated by dividing the area of the resected specimen by the procedure time (cm^2/min). The approximate oval area (cm^2) of the resected specimen was calculated as follows; 3.14 × 0.25 × long axis diameter × short axis diameter.

Adverse events

Perforation and postoperative bleeding were counted as adverse events related to the procedure.

Postoperative bleeding was recorded if one of the following conditions was identified: (1) bleeding that required endoscopic hemostatic treatment; and (2) bleeding with a reduction in total hemoglobin of more than 2 g/dL from the preoperative level. Perforation was diagnosed by endoscopic findings during ESD or by the presence of free air on computed tomography.

Ethics

All patients were informed of the risks and benefits of ESD, and provided written informed consent to undergo the procedure. This study was approved by the Ethics Committees of Kobe University Hospital (No. 160051) and Kishiwada Tokushukai Hospital (No. 28-10).

Statistical analysis

The Mann-Whitney *U* test was used to compare continuous variables, and the χ^2 test or Fisher's exact probability test was used to compare categorical variables. $P < 0.05$ was considered significant. All statistical analyses were performed using JMP version 10 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Results of functional experiments

The results of the functional experiments revealed that water aspiration speed by the endoscope equipped

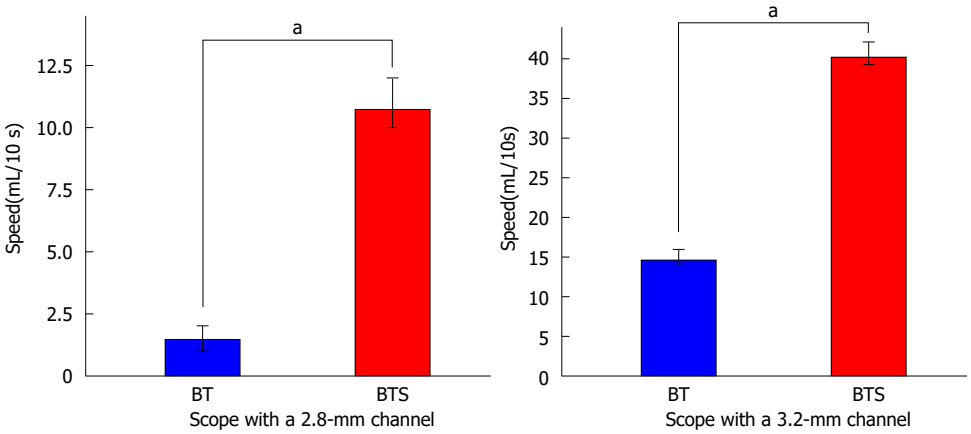

Figure 2 Water aspiration speed. A total of 200 mL water in a graduated cylinder was aspirated by scopes with 2.8-mm and 3.2-mm channels with FlushKnife-BT or FlushKnife-BTS inserted, and the amount of aspirated water in 10 s was measured. The column denotes mean data and the bar shows the range of experiments performed 9 times. Water aspiration speed was markedly faster with FlushKnife-BTS than with FlushKnife-BT. [a]*P* < 0.05, BT *vs* BTS. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

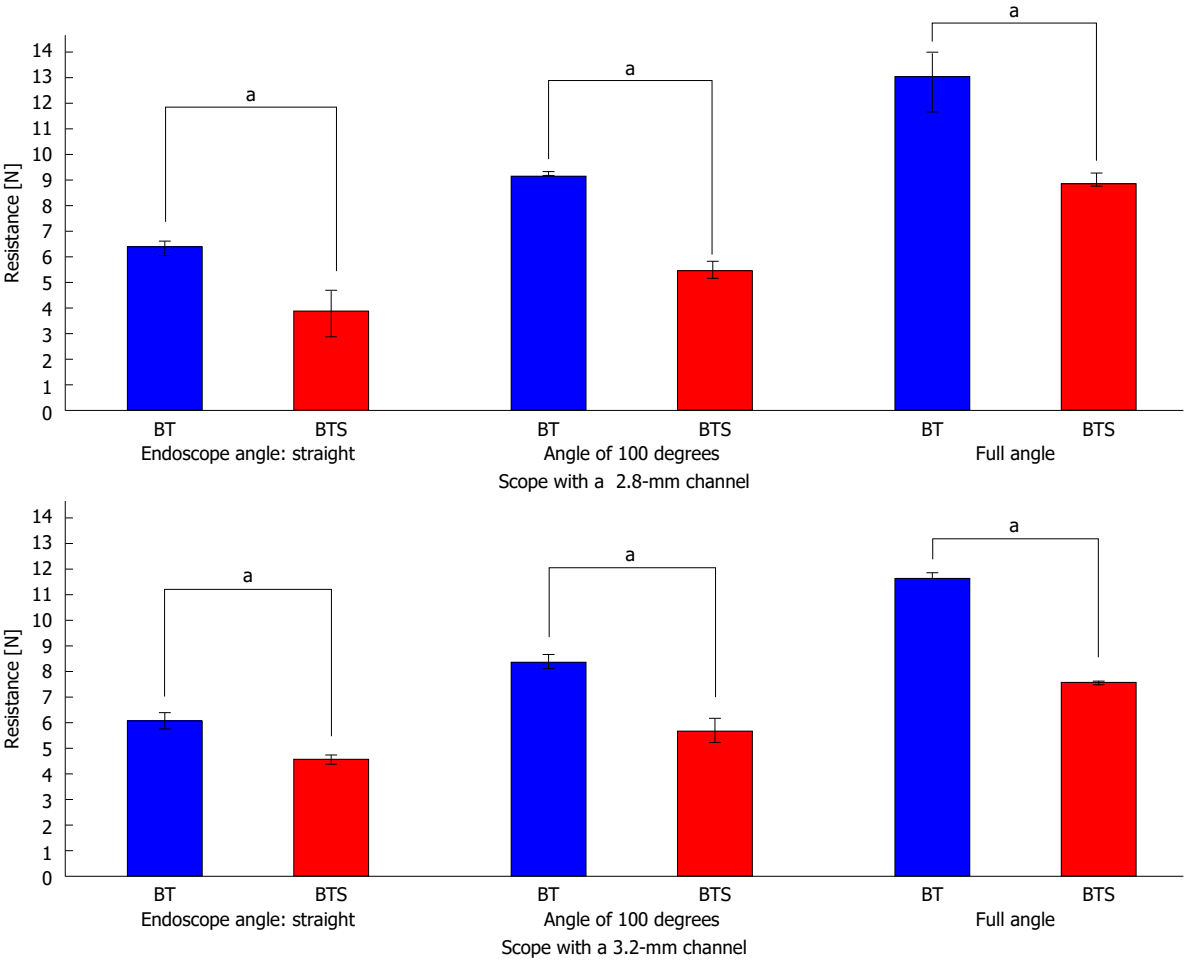

Figure 3 Resistance to knife insertion inside the scope. Resistance to the insertion of Flushknife-BT or Flushknife-BTS inside the scope at various endoscopic angles was measured using a measuring instrument by NIDEC-SHIMPO CORPORATION. The column denotes mean data and the bar shows the range of experiments performed in triplicate. Resistance was lower at all endoscopic angles with Flushknife-BTS than with Flushknife-BT. [a]*P* < 0.05, BT *vs* BTS. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

with a 2.8-mm working channel with FlushKnife-BTS inserted was 7.7-fold faster than that with FlushKnife-BT (Figure 2). Even when using an endoscope with a 3.2-mm working channel, water aspiration speed was 2.7-fold faster with FlushKnife-BTS than with FlushKnife-BT. Resistance to knife insertion through the 2.8-mm working channel in a straight endoscopic position was 40% less using the new slim knife than

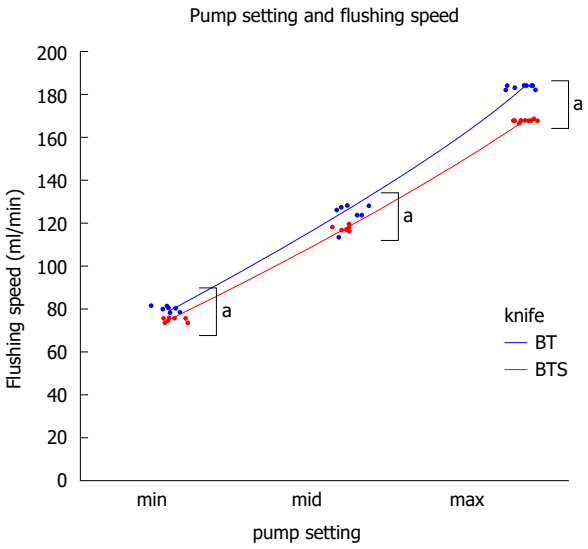

Figure 4 Waterjet flushing speed. Waterjet flushing speed was faster with FlushKnife-BT than with FlushKnife-BTS at all pump settings tested. [a]*P* < 0.05, BT *vs* BTS. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

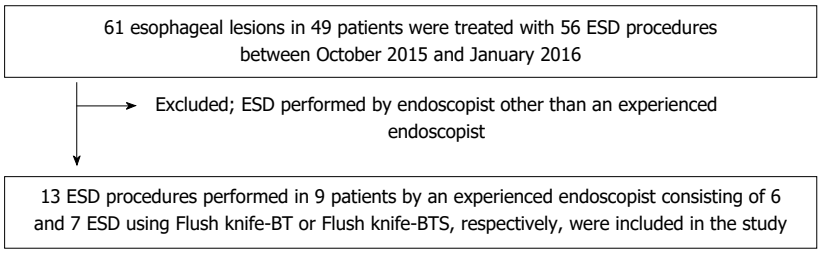

Figure 5 Flow diagram of cases that underwent endoscopic submucosal dissection included in the analysis. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT; ESD: Endoscopic submucosal dissection.

Table 1 Patient and lesion characteristics

	FlushKnife-BT (*n* = 6)	FlushKnife-BTS (*n* = 7)	*P* vaule[1]
Male/Female	3[2]/1[2]	4[2]/1	0.559
Age, median (range), years	73.5 (68-78)	57 (57-74)	0.0235
Lesion site Ut/Mt/Lt	0/4/2	0/6/1	0.559
Circumference of the resected area, median (range)	68% (25-100)	75% (50-92)	0.599
Major axis diameter of the resected specimen, median (range), mm	39.5 (30-55)	40 (31-60)	0.277
Resected area, median (range), mm²	1117 (636-2547)	1193 (511-2418)	0.943
Major axis diameter of the tumor[3], median (range), mm	33 (19-42)	32 (25-50)	0.616
Histology of the tumor HGIN/SCC	0/6	0/7	0.000
Depth of the tumor EP/LPM/MM	0/5/1	1/5/1	0.629

[1]The Mann-Whitney *U* test was used to compare continuous variables, and the chi-squared test or Fisher's exact probability test was used to compare categorical variables; [2]One patient had multiple esophageal lesions treated by endoscopic submucosal dissection; [3]In the case of multiple lesions resected simultaneously, the maximum distance between the edges of each tumor was defined as the major axis diameter of the tumor. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

the conventional knife (Figure 3). Reductions in resistance were detected using both scopes with 2.8-mm and 3.2-mm working channels and at any endoscopic angle. Waterjet flushing speed was lower with FlushKnife-BTS than with FlushKnife-BT at all water pressure settings tested (Figure 4). This difference became larger with increases in water pressure.

Clinical results

During the study period, 61 esophageal lesions in 49 patients were treated with 56 ESD procedures. Of these, 13 ESD procedures performed in 9 patients, consisting of 6 and 7 ESD using FlushKnife-BT and FlushKnife-BTS, respectively, were completed by an experienced endoscopist and these cases were

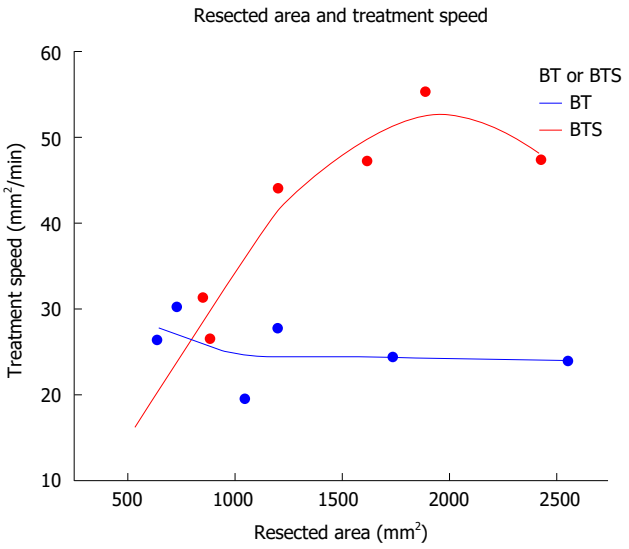

Figure 6 Treatment speed was faster with a novel slim type ball-tipped FlushKnife when the resected size was large, but was similar to that with the conventional knife when the resected size was small. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

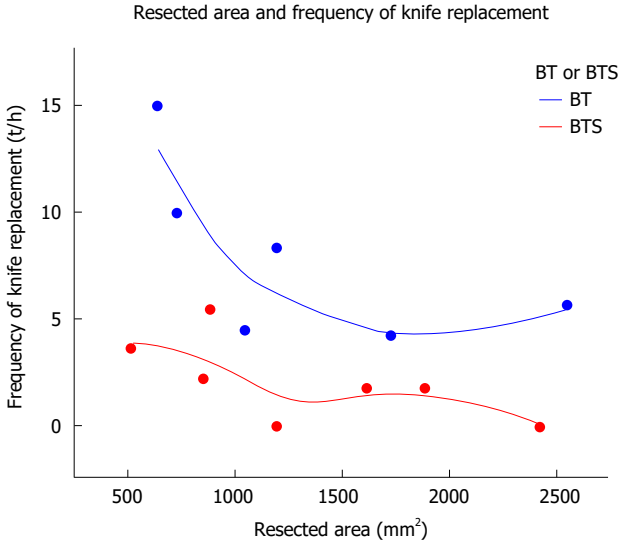

Figure 7 Frequency of knife replacement was reduced with a novel slim type ball-tipped FlushKnife regardless of the resected size, while a slightly reduced frequency was observed as the resected size became larger. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

included in the study (Figure 5).

The characteristics of the patients and lesions are shown in Table 1. There were 3 males and 1 female in the BT group, and 4 males and 1 female in the BTS group. Two patients in the BT group and one patient in the BTS group had multiple lesions that were treated separately. Ages ranged between 68 and 78 years with a median age of 73.5 years in the BT group, and between 57 and 74 years with a median age of 57 years in the BTS group (P = 0.0235). Lesion sites (Ut/Mt/Lt) were 0/4/2 and 0/6/1, respectively (P = 0.559).

The circumference of the resected area ranged between 25% and 100% with a median of 68% in the BT group, and between 50% and 92% with a median of 75% in the BTS group (P = 0.599).

The median major axis diameters of resected specimens were 39.5 mm (range 30-55) and 40 mm (range 31-60) respectively (P = 0.277) in BT and BTS groups, respectively.

The median resected area was 1117 mm^2 (range 636-2547) in the BT group and 1193 mm^2 (range 511-2418) in the BTS group (P = 0.943).

The median major axis diameter of tumors was 33 mm (range 19-42) in the BT group and 32 mm (range 25-50) in the BTS group (P = 0.616). In the case of multiple lesions resected together, the maximum distance between the edges of each tumor was defined as the tumor size.

The histology of tumors revealed SCC in all cases. The depths of tumors (EP/LPM/MM) were 0/5/1 in the BT group and 1/5/1 in the BTS group (P = 0.629). The outcomes of ESD are shown in Table 2. The thread-traction method was performed on 3 out of 6 cases in the BT group and 2 out of 7 cases in the BTS group (P = 0.592). Median procedure times were 48 min (range 24-106) and 33 (range 27-51) min in the BT and BTS groups, respectively (P = 0.389).

The median treatment speed was 25.5 mm^2/min (range 19.6-30.3) in the BT group and 44.2 mm^2/min (range 15.5-55.4) in the BTS group (P = 0.0633). However, it was significantly faster with FlushKnife-BTS when the resection size of ESD was larger than 1000 m^2 (n = 4, median 24.2, range 19.6-27.7 *vs* n = 4, median 47.4 mm^2/min, range 44.2-55.4, P = 0.0209) (Table 3). The relationship between the resected area and treatment speed is shown in Figure 6; the treatment speed was faster with FlushKnife-BTS when the resected size was large, but was similar to that with FlushKnife-BT when it was small.

The number of times the knife was replaced was 5.5 (range 4-10) and 1 (range 0-3) in the BT and BTS groups, respectively (P = 0.0025).

The frequency of knife replacement was lower (median 1.76, range 0-5.45 *vs* median 7.02, range 4.23-15 times in one hour, P = 0.0065) with FlushKnife-BTS than with FlushKnife-BT. Figure 7 shows the relationship between the resected area and frequency of knife replacement, and demonstrates a reduced frequency of knife replacement with FlushKnife-BTS regardless of the resected size and slightly reduced frequency as the resected size became larger. *En bloc* resection rates were 100% and muscle injury was detected in one case in the BT group.

DISCUSSION

ESD is the first-line therapeutic option for superficial gastrointestinal tract tumors[1,6,18-20]. The devices used in ESD are important for performing the procedure safely. A large number of devices have been developed

Table 2 Outcomes of endoscopic submucosal dissection by novel slim type ball-tipped FlushKnife and ball-tipped FlushKnife

	FlushKnife-BT (n = 6)	FlushKnife-BTS (n = 7)	P vaule[1]
Use of the thread-traction method, yes/no	3/3	2/5	0.592
Procedure time, median (range), min	48 (24-106)	33 (27-51)	0.389
Treatment speed, median (range), mm^2/min	25.5 (19.6-30.3)	44.2 (15.5-55.4)	0.0633
Number of times the knife was replaced, median (range), times	5.5 (4-10)	1 (0-3)	0.0025
Frequency of knife replacement, median (range), times/hour	7.02 (4.23-15)	1.76 (0-5.45)	0.0065
En bloc resection rate	6/6 (100%)	7/7 (100%)	0.000
Adverse events, Perforation/muscle injury/postoperative bleeding	0/1/0	0/0/0	0.462

[1]The Mann-Whitney U test was used to compare continuous variables, and the χ^2 test or Fisher's exact probability test was used to compare categorical variables. FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

Table 3 Treatment speed of endoscopic submucosal dissection for a resected size more than 1000 mm^2

	FlushKnife-BT (n = 4)	FlushKnife-BTS (n = 4)	P vaule
Treatment speed, median (range), mm^2/min	24.4 (19.6-27.7)	47.4 (44.2-55.4)	0.0209

FlushKnife-BT: Ball-tipped FlushKnife; FlushKnife-BTS: A novel slim type FlushKnife-BT.

to date[8-10,12,13]. FlushKnife-BT is one of the most frequently used knives and has advantageous functions including injection, irrigation by waterjets, dissection, and vessel sealing[14]. However, when this knife is used with endoscopes equipped with a 2.8-mm channel, difficulties are associated with the aspiration of fluid and mucus in the working space during the procedure and finely controlling the knife length because of its diameter of 2.7 mm. Uncontrolled fluid and mucus pooling and air inflation/deflation may complicate ESD. In order to precisely dissect the appropriate plane between the vessel network in the submucosa and muscle layer[21], subtle endoscope movements in addition to adjustments in knife length are needed. Therefore, smooth aspiration and delicate knife control are essential for performing this procedure safely and efficiently.

In an attempt to overcome these limitations, we developed a novel slim type FlushKnife-BT.

Functional experiments revealed that fluid aspiration speed by the endoscope with a 2.8-mm working channel with FlushKnife-BTS inserted was 7.7-fold faster than that with the conventional knife. Resistance to the insertion of the knife inside the scope with a 2.8-mm working channel was 40% less with the new knife than with the conventional knife.

In clinical practice, though the number of the patients was small, increase was achieved in the treatment speed with FlushKnife-BTS when large resection was required, but remained the same as that with the conventional knife when the resected size was small. The frequency of knife replacement was less with FlushKnife-BTS than with FlushKnife-BT regardless of the resected size.

The faster aspiration of bubbles, air, mucus, and fluid by FlushKnife-BTS contributed to a clear field of view, which may have, in turn, reduced the frequency of knife removal from the working channel.

The reason why treatment speed only improved with FlushKnife-BTS when the resection size was large may be that, in ESD of a small resection size, the effects by a reduced frequency of knife replacement, smooth knife insertion, and fine knife control with FlushKnife-BTS were not clearly reflected due to the short procedure time and fewer knife replacements, but became more evident as the resection size became larger and the procedure time increased. Therefore, FlushKnife-BTS is considered to exhibit its effectiveness when large resection is needed.

Waterjet flushing speed was slower with FlushKnife-BTS in functional experiments. This result was expected due to the difference in the diameters of the two knives. However, this did not markedly affect the clinical practice of ESD in our analysis. Since FlushKnife-BT offers a high-flow flushing function, the speed reductions observed with FlushKnife-BTS do not appear to be of clinical importance. Moreover, the mid pump setting is typically used in clinical practice and may be resolved by turning up the setting to its maximum where necessary.

Although the safety and efficacy of ESD in the esophagus have already been reported[2,6,22], intraoperative perforation, muscle layer damage, and bleeding may occur because of the anatomically thin wall and narrow working space. Moreover, postoperative esophageal stricture is one of the main complications associated with large esophageal ESD[23,24]. Muscle layer damage with entire circumferential esophageal ESD has been linked to refractory post-ESD stenosis[25]. Hence, ESD in the esophagus requires a highly skilled endoscopic technique and careful operation, and the provision of a more comfortable environment for ESD will contribute to reductions in complications. Based on the results presented above, FlushKnife-BTS is considered to contribute to safer esophageal ESD.

Though the present clinical study focused on only esophageal ESD because esophagus is the narrow

tract in which inflated air inside affects the procedure easily, further investigation including ESD in other organs such as stomach and colorectum would be desired in the near future.

The limitations of this study include its retrospective design and small patient population. Furthermore, the procedure was only performed by one endoscopist. Therefore, the generalizability of the results obtained remains unclear and, thus, further studies with more cases undergoing ESD performed by other endoscopists including less experienced operators are warranted. However, our results still support FlushKnife-BTS creating better conditions for and contributing to the efficient performance of ESD.

In conclusion, our results demonstrate that FlushKnife-BTS supports the efficient performance of ESD, particularly for large lesions, by improving air and fluid aspiration and allowing for smooth knife insertion without frequent knife removal and reinsertion during ESD.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) has been widely accepted as a treatment for early-stage tumors in the digestive tract. Devices utilized in ESD play an important role in facilitating the safe and effective performance of this procedure. A novel slim type ball-tipped FlushKnife (FlushKnife-BTS) has been developed to enhance the performance of aspiration and insertion of the knife through the scope.

Research frontiers

This study investigated the usefulness of FlushKnife-BTS over FlushKnife-BT in functional experiments and clinical practice and is the first report comparing the conventional ESD knife and the developed new one.

Innovations and breakthroughs

This study indicated that FlushKnife-BTS enhances the performance of ESD, particularly for large lesions, by improving air and fluid aspiration and knife insertion during ESD and reducing the frequency of knife removal and reinsertion.

Applications

This study suggested that FlushKnife-BTS supports the efficient performance of ESD, particularly for large lesions.

Terminology

FlushKnife-BTS is a novel slim type FlushKnife BT that has been developed to enhance the performance of aspiration and insertion of the knife through the scope.

Peer-review

Theoretically, the new device facilitates use in a standard scope with 2.8 mm of working channel. The paper compares a new and an older device with respect to the ability of insertion and suction in the laboratory as well as the resection speed by the measurement of the mm^2 per minute in esophageal lesions in clinical practice.

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ORIGINAL ARTICLE

Prospective Study

Spectral computed tomography in advanced gastric cancer: Can iodine concentration non-invasively assess angiogenesis?

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Abstract

AIM

To investigate the correlation of iodine concentration (IC) generated by spectral computed tomography (CT) with micro-vessel density (MVD) and vascular endothelial growth factor (VEGF) expression in patients with advanced gastric carcinoma (GC).

METHODS

Thirty-four advanced GC patients underwent abdominal enhanced CT in the gemstone spectral imaging mode. The IC of the primary lesion in the arterial phase (AP) and venous phase (VP) were measured, and were then normalized against that in the aorta to provide the normalized IC (nIC). MVD and VEGF were detected by immunohistochemical assays, using CD34 and VEGF-A antibodies, respectively. Correlations of nIC with MVD, VEGF, and clinical-pathological features were analyzed.

RESULTS

Both nICs correlated linearly with MVD and were higher in the primary lesion site than in the normal control site, but were not correlated with VEGF expression. After stratification by clinical-pathological subtypes, nIC-AP showed a statistically significant correlation with MVD, particularly in the group with tumors at stage T4, without nodular involvement, of a mixed Lauren type, where the tumor was located at the antrum site, and occurred in female individuals. nIC-VP showed a positive correlation with MVD in the group with the tumor at stage T4 and above, had nodular involvement, was poorly differentiated, was located at the pylorus site, of a mixed and diffused Lauren subtype, and occurred in male individuals. nIC-AP and nIC-VP showed significant differences in terms of histological differentiation and Lauren subtype.

CONCLUSION

The IC detected by spectral CT correlated with the MVD. nIC-AP and nIC-VP can reflect angiogenesis in different pathological subgroups of advanced GC.

Key words: Micro-vessel density; Iodine concentration; Spectral computed tomography; Vascular endothelial growth factor; Gastric cancer

Core tip: We investigated the correlation between iodine concentration (IC) value generated from spectral computed tomography (CT) and angiogenesis in gastric cancer (GC) with clinical-pathological data. Our results showed that normalized IC (nIC) in both the arterial (AP) and venous phases (VP) had a positive linear correlation with micro-vessel density. nIC-AP reflected the angiogenesis in relatively earlier and well-differentiated GC, while nIC-VP reflected this in further advanced and poorly differentiated GC. Spectral CT with quantitative IC value offers a new choice for evaluating the angiogenesis of gastric cancer noninvasively.

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INTRODUCTION

Despite a recent decrease, gastric cancer (GC) remains the most common cancer and is the third leading cause of cancer-related death globally[1]. In China, the incidence and mortality rates of GC remain high, and the vast majority of cases are in the advanced stage[2], which requires more attention.

Angiogenesis is fundamental to the growth, invasion, and metastasis of GC, and greatly influences the response to anti-tumor therapies[3]. To date, the standard method for studying angiogenesis has been histopathological counting of micro-vessel density (MVD), which is specimen- and immunostaining-dependent. This is impractical in advanced patients undergoing anti-angiogenesis or chemotherapy. Compared with MVD counting, using preoperative imaging modalities for the noninvasive assessment of tumor angiogenesis is more acceptable and feasible. A previous study has revealed that MVD, vascular endothelial growth factor (VEGF), and the absolute enhanced value show some positive correlations with conventional contrast-enhanced computed tomography (CT)[4]. Additionally, CT perfusion has shown the potential for evaluating tumor angiogenesis[5,6], but the complicated measurement and high radiation dosage have limited the extensive application of CT in this regard.

The recently developed spectral CT yields material-decomposition (MD) images that can quantitatively map the iodine concentration (IC) of the tissue in enhanced images. This IC value has been proven to show a strong correlation with the actual iodine concentration in the phantom[7]. Recently, preliminary studies have reported the use of the IC value to differentiate benign and malignant lesions, to find embolisms, and to evaluate the efficacy of anticancer therapy[8-11]. Particularly in the evaluation of neoadjuvant chemotherapy of GC, the IC value was proven to be a more robust imaging biomarker than the morphology index[11]. However, it was not clear how this correlated with radiological-pathological features. On the other hand, the IC value has shown a correlation with vascularization in solid tumors, such as pancreatic carcinoma, hepatocarcinoma, and non-small-cell lung cancer[12-14], while there has been no such report for GC, to provide a basis for using image indicators, other than morphology, for assessing chemo-efficacy. Therefore, our purpose was to investigate the correlation between IC value and angiogenesis in GC cases with clinical-pathological data.

MATERIALS AND METHODS

Study population

Adult patients with advanced GC confirmed by endoscopic biopsy, who were scheduled for surgery, were enrolled. This study was approved by the institutional review board, and informed consent was obtained from each participant. All procedures were performed in accordance with the ethical standards of the institution.

Exclusion criteria were: (1) allergies to intravenous contrast media; (2) cardiac or renal insufficiency; (3) history of chemotherapy or radiotherapy; (4) inability to visualize the tumor on CT; (5) early tumor staging (T1) or presence of distant metastasis (M1); and (6)

specimen with poor fixation for immunostaining.

From June 2014 to May 2015, a total of 41 patients prospectively underwent spectral CT examination. Of these, two patients with serious interface artifacts on CT images, one patient with tumor tissue necrosis that influenced MVD counting, one patient with no tumor cells on hematoxylin and eosin (HE) slices, and three patients with failed immunostaining were excluded.

Ultimately, the data of 34 patients were collected and statistically analyzed. Patient records and pathological data, including gender, age, tumor size, tumor location, invasion depth, lymph nodes involvement, Lauren subtypes, and differentiation, were documented.

CT scan methods

After fasting overnight, all patients were administered 10 mg anisodamine (Minsheng Pharmaceutical Group Co., Ltd., Hangzhou, China) intramuscularly to reduce gastrointestinal motility 20 min prior to CT examination, and ingested 800-1000 mL of water to distend the stomach. During scanning, patients were instructed to suspend respiration.

All examinations were performed on a Discovery CT750 HD system (GE Healthcare, Milwaukee, WI, United States), and included bi-phasic enhanced spectral scanning in the arterial and venous phases (AP and VP, respectively). Spectral CT imaging was performed with a 0.5 ms switch of tube voltage between 140 kVp and 80 kVp; a rotation speed of 0.6 s, and a helical pitch of 1.375:1. The scan range was from the diaphragmatic dome to the symphysis pubis. Non-ionic contrast material, iohexol (350 mg I/mL, GE Pharmaceutical, Shanghai of China), at 1.3 mL per kilogram of body weight was used (total volume: 60-110 mL) at a flow rate of 2.5-4.5 mL/s was injected *via* a peripheral vein, using a dual high-pressure syringe. The AP acquisition time was triggered at 9 s after the attenuation of diaphragmatic abdominal aorta reached 100 HU (SmartPrep; GE Healthcare). The VP followed with a 30 s interval. Raw data were reconstructed to 1.25-mm slice images, using decompose projection-based software. An additional 40% adaptive statistical iterative reconstruction algorithm was applied to suppress image noise and decrease the radiation dose required for spectral CT.

Image analysis

All CT images were transferred to a commercially available workstation (Advantage Windows 4.6; GE Medical Systems, Chicago, IL, United States) to generate iodine-based MD images (Figures 1 and 2). Two experienced radiologists (J.G. and P.L., with 25 and 5 years of experience with abdominal CT, respectively), who were blinded to the pathological results, analyzed the images. Three manually drawn regions of interests (ROIs) that encompassed the maximum lesion in the consecutive 1.25-mm layers of bi-phasic axial images were measured. Areas containing prominent artifacts, necrosis, and vessels were carefully avoided (mean square: 871 mm^2; range: 122-1308 mm^2). For the normal site, we chose a distance longer than 5 cm from the lesion edge that was thick enough to place the ROI. Three small circular ROIs with a diameter exceeding 2 mm were measured as ROI-normal. Images were compared to ensure the measurements were as consistent as possible in both phases. The ICs in the AP (IC-AP) and in the VP (IC-VP) were generated simultaneously (unit: 100 μg/mL). At the same time, a round ROI was placed on the abdominal aorta in the same layer as the target, to calculate the normalized IC (nIC = IC_{target}/IC_{aorta})[15], aiming to reduce the individual circulatory variability. All the ICs and nICs obtained from the same patient were averaged and disagreement on measurement was resolved by consensus.

Histopathologic evaluation

A surgeon (K.R) and a radiologist (X.C) performed sampling together to guarantee that the specimen and the ROI were from virtually the same level, by re-examining the axial and multi-planar reconstruction images. The distance to the cardia or pylorus of the sample site and the thickness were compared on both CT images and surgical specimens. Then, the selected samples (tumor and normal wall) were fixed overnight in 10% formalin, and subsequently dehydrated in alcohol and paraffin-embedded. Wax blocks comprising the central part of the adenocarcinoma were sectioned into 4-μm-thick slices. A ready-to-use two-step streptavidin-peroxidase method was applied. The mouse anti-CD34 monoclonal antibody (diluted 1:150) and rabbit anti-VEGF-A polyclonal antibody (diluted 1:200) (Beijing Zhongshan Goldenbridge Company, Beijing, China) were used to stain all pathological tissues. Positive and negative immunohistochemistry controls were prepared as routine. A pathologist (K.C, with 25 years' experience in MVD and VEGF immunostaining), who was blinded to the spectral CT imaging results, performed the counting and evaluation of all slides.

MVD counting was performed using the Weidner method[16]. The area where vascular endothelial cells stained most intensively was first identified at low (× 100) magnification. Then, five fields were randomly selected at × 400 magnification to count the CD34-positive cell clusters and the mean was recorded as the final value (Figures 1 and 2). VEGF (stained brown), located in the cytoplasm, was scored using an established method[17]: scores of the percentage of positive tumor cells and signal intensity were summed. If the score was less than 4, the section was considered negative, whereas a score of 4 or more was considered positive (Figures 1 and 2).

Statistical analysis

All statistical procedures were performed with a software package (SPSS 21.0, Chicago, IL, United States).

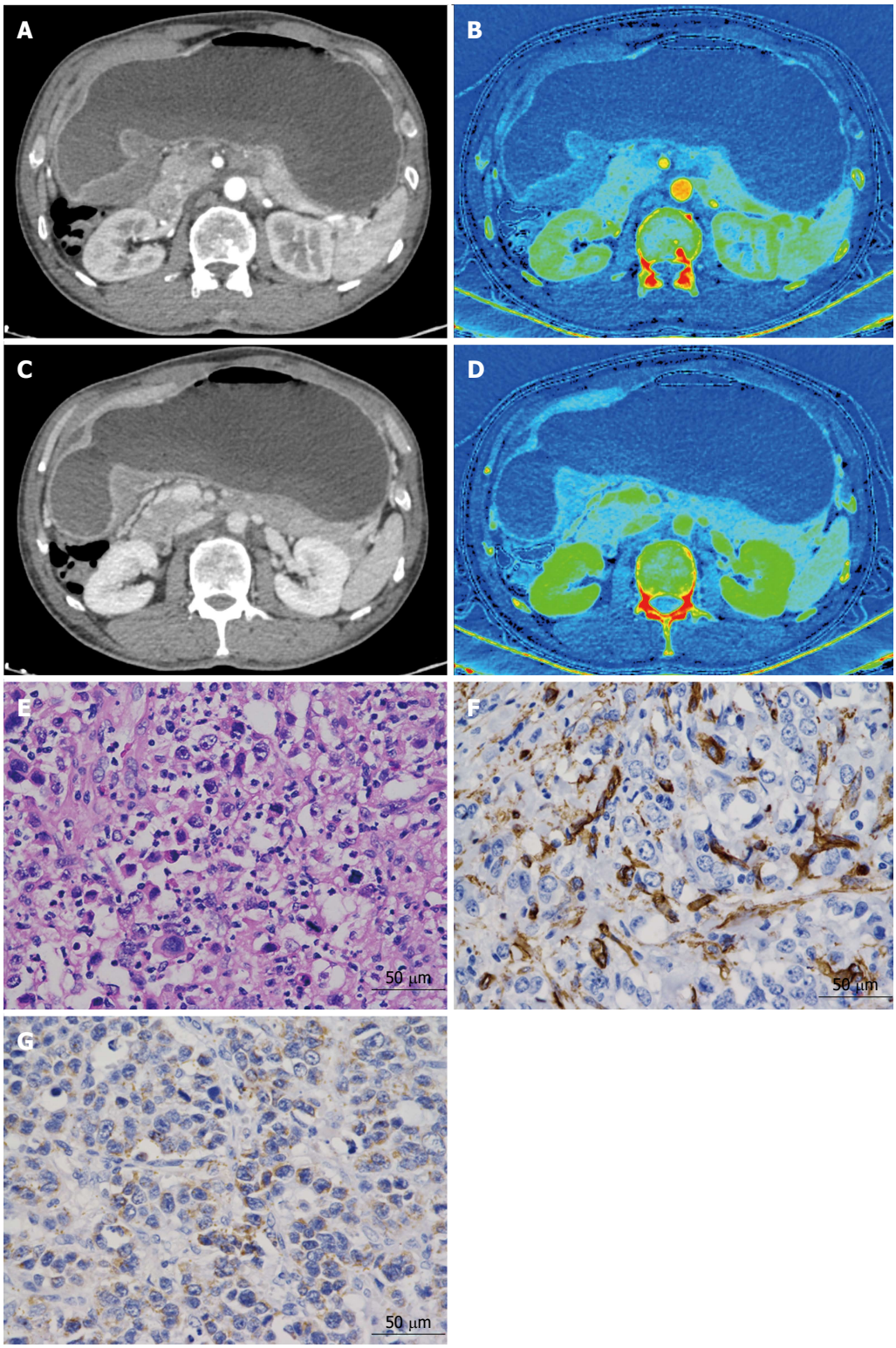

Figure 1 Detection of the iodine concentration value in a 51-year-old man with poorly differentiated adenocarcinoma, with staging Ⅲc (T4aN3M0). A: The monochromatic image shows focal wall thickening in the gastric antrum; B: The iodine-water image with iodine concentration (IC) value 12.83 (100 μg/cm^3), normalized IC (nIC) value 0.11 in the arterial phase. Monochromatic image (C) and iodine-water image (D) with IC value 23.91 (100 μg/cm^3), nIC value 0.53 in the venous phase; E: Hematoxylin and eosin staining of a pathological section obtained from radical surgery shows poorly differentiated, diffused subtype in the Lauren classification (× 400); F: CD34-staining shows endothelial cells stained brown; micro-vessels form clusters or have tiny hollow lumens (micro-vessel density 45/magnification × 400). G: Weak vascular endothelial growth factor staining in the cytoplasm (× 400) with score 2.

A *P*-value of less than 0.05 was considered to indicate a statistically significant difference. Nodal status was classified as positive and negative for lymph node metastasis, and the depth of invasion was classified as positive and negative serosal involvement. A single highly differentiated patient was grouped together

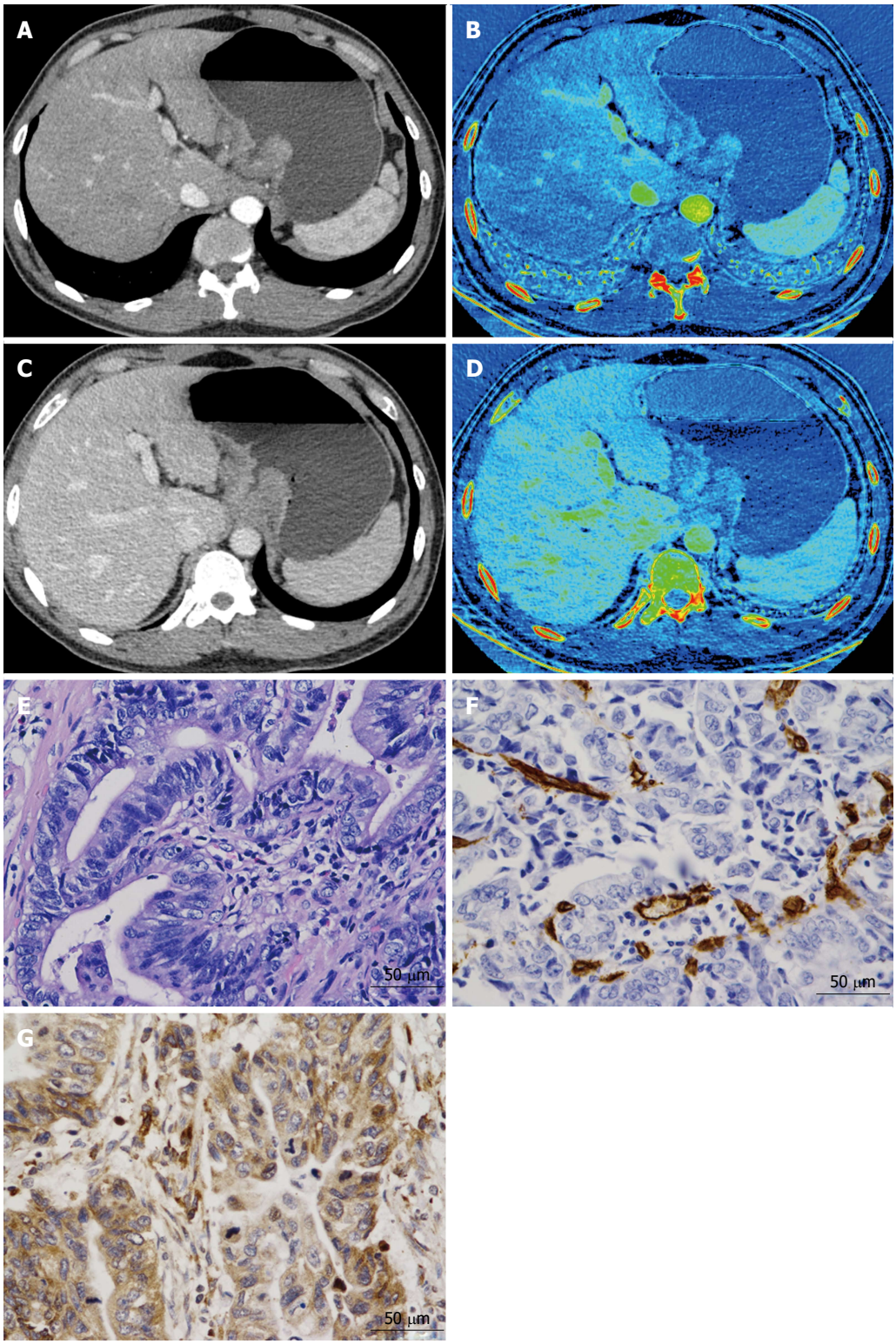

Figure 2 Detection of iodine concentration value in a 50-year-old man with moderate differentiated adenocarcinoma, of staging Ⅲa (T4aN1M0). A: Monochromatic image shows focal wall thickening in the gastric cardia and lesser curvature; B: The iodine-water image with iodine concentration (IC) value 11.78 (100 μg/cm^3), normalized IC (nIC) value 0.09 in the arterial phase; Monochromatic image (C) and (D) iodine-water image with IC value 18.63 (100 μg/cm^3), nIC value 0.34 in the venous phase; E: Hematoxylin and eosin staining shows a moderately differentiated, intestinal Lauren subtype (× 400); F: Immunohistochemical staining shows CD34 positive micro-vessel (micro-vessel density count: 27/magnification × 400); G: Strongly positive vascular endothelial growth factor staining (× 400) with score 5.

with the moderately differentiated patients. Data were subjected to a Kolmogorov-Smirnov normality test and continuous variables were presented as means and standard errors of the mean. When analyzing the correlation of nIC and MVD and VEGF, scatter plots were made first between continuous variables, followed by the Pearson or the Spearman rank-correlation test (Figure 3). Student's *t*-test, correct *t* test, and one-way

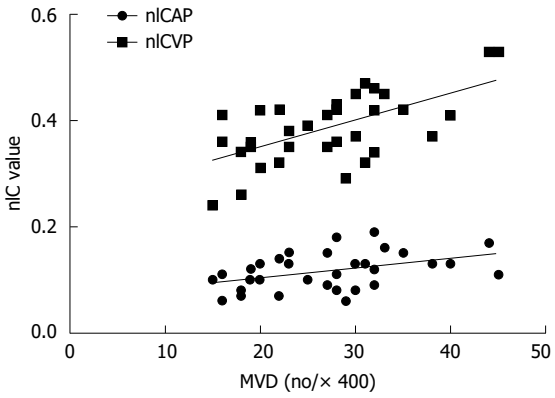

Figure 3 Scatter plots of normalized iodine concentration in arterial phase, normalized iodine concentration in venous phase, and microvessel density counts in tumor lesions (r = 0.423 for normalized iodine concentration in arterial phase, r = 0.606 for normalized iodine concentration in venous phase). VEGF: Vascular endothelial growth factor; nIC: Normalized iodine concentration; nIC-AP: nIC-arterial phase; nIC-VP: nIC-venous phase; MVD: Microvessel density.

analysis of variance (ANOVA)-LSD test were performed to analyze differences in categorical data between groups (serosal involvement, lymph node metastasis, histologic differentiation, Lauren subtype, tumor location, and gender).

RESULTS

A total of 34 advanced GC patients (23 males, 11 females; mean age: 56 ± 3.7 years; age range: 30-73 years) were included (Table 1). Tumor size ranged from 1.5 cm to 13.0 cm (median: 4.0 cm). Fourteen tumors located in the gastric cardia-fundus, nine in the gastricum, and 11 in antrum. All patients were treated surgically by radical gastrectomy and D2 lymph node dissection. Based on pathologic results, the adenocarcinoma was well differentiated in one patient, moderately differentiated in 16 patients, and poorly differentiated in 17 patients. According to the 7th American Joint Committee on staging classification (AJCC), five patients were classified as T2, two as T3, twenty-two as T4a and five as T4b; eleven as N0, eight as N1, seven as N2, and eight as N3. No patient had distant metastasis. The Lauren classification was as follows: 15 were intestinal type, seven were mixed type, and 12 were diffuse type. Additionally, the immunostaining analysis revealed that 25 patients (73.53%) stained positive for VEGF with a score of 4 (n = 11), 5 (n = 9), or 6 (n = 5). The mean MVD count for these 34 tumors was 26.94 ± 1.35.

In general, the bi-phasic nIC values and the MVD counts were positively correlated (P = 0.013, P < 0.001, respectively). VEGF did not correlate with either nIC value or with MVD, as shown in Table 2. When stratified by different clinical features, the correlation coefficient value increased. nIC-AP positively correlated with MVD in patients with tumor stage less than T4 (r = 0.851, P = 0.015), tumor of N0 stage (r = 0.620, P = 0.042), with a tumor located in the pylorus region (r = 0.616, P = 0.044), and who were female (r = 0.696, P = 0.017). On the other hand, nIC-VP correlated with MVD in the more advanced group of patients, with tumors above T4 stage (r = 0.656, P < 0.001), with nodular involvement (r = 0.644, P = 0.001), that were poorly differentiated (r = 0.799, P < 0.001), were of mixed and diffused Lauren subtypes (r = 0.827, P = 0.022; r = 0.765, P = 0.004, respectively), and male gender (r = 0.606, P = 0.002), as shown in Table 3.

The nIC values in the primary GC and normal gastric wall were 0.116 ± 0.033 and 0.101 ± 0.023 in arterial phase (P = 0.033), 0.386 ± 0.061, and 0.286 ± 0.066 in the venous phase (P < 0.001) (Figure 4A). For the VEGF-positive and -negative group, neither nIC-AP nor nIC-VP showed statistically significant differences (Figure 4B).

When stratified by clinical subgroups, both nIC-AP and nIC-VP were higher in patients with serosal involvement, lymph node metastasis, poor differentiation, and diffused Lauren type than in those with depth invasion under T4, nodal status N0, high and moderate differentiation, intestinal and mixed Lauren type, but these differences did not reach statistical significance.

Table 1 Clinical characteristics of the patients (n = 34)

Sex	Male	23	67.65%
	Female	11	32.35%
Age	30 -73 yr (56 ± 3.7 yr)		
Size	1.5 -13.0 cm (median 4.0 cm)		
Tumor location	Cardia/Fundus	14	41.18%
	Gastricum	9	26.47%
	Antrum	11	32.35%
Nodal status	N0	11	32.35%
	N1	8	23.53%
	N2	7	20.59%
	N3	8	23.53%
Depth of invasion	pT2	5	14.71%
	pT3	2	5.89%
	pT4a	22	64.71%
	pT4b	5	14.71%
Lauren subtype	Intestinal type	15	44.12%
	Mixed type	7	20.59%
	Diffuse type	12	35.29%
Histological grading	Highly differentiated	1	2.38%
	Moderately differentiated	16	47.06%
	Poorly differentiated	17	50.00%

Table 2 Correlation of nIC values, microvessel density and vascular endothelial growth factor expression

Varieties	MVD		VEGF	
	r	P value	r	P value
nIC-AP	0.423	0.013[a]	0.170	0.358
nIC-VP	0.606	0.000[a]	0.311	0.073
MVD	1.000		0.210	0.233

[a]P < 0.05. nIC-AP: Normalized iodine concentration in arterial phase; nIC-VP: Normalized iodine concentration in venous phase; MVD: Microvessel density; VEGF: Vascular endothelial growth factor.

Table 3 Correlations between bi-phase normalized iodine concentration and microvessel density in different clinical-pathological subgroups

Varieties	*n*	nIC-AP *vs* MVD		nIC-VP *vs* MVD	
		r	*P* value	*r*	*P* value
Depth of invasion					
< T4	7	0.851	0.015[a]	0.600	0.154
T4	27	0.370	0.057	0.656	0.000[a]
Nodal status					
N0	11	0.620	0.042[a]	0.600	0.051
N1-3	23	0.330	0.124	0.644	0.001[a]
Histologic differentiation					
Highly and Moderately differentiated	17	0.250	0.334	0.190	0.466
Poorly differentiated	17	0.427	0.087	0.799	0.000[a]
Lauren subtype					
Intestinal type	15	0.222	0.427	0.101	0.719
Mixed type	7	0.741	0.057[a]	0.827	0.022[a]
Diffuse type	12	0.145	0.653	0.765	0.004[a]
Tumor location					
Cardia/Fundus	14	0.311	0.279	0.760	0.796
Gastricum	9	0.385	0.307	0.507	0.163
Antrum	11	0.616	0.044[a]	0.891	0.000[a]
Sex					
Male	23	0.385	0.070	0.606	0.002[a]
Female	11	0.696	0.017[a]	0.605	0.049

[a]$P < 0.05$. nIC-AP: Normalized iodine concentration in arterial phase; nIC-VP: Normalized iodine concentration in venous phase; MVD: Microvessel density.

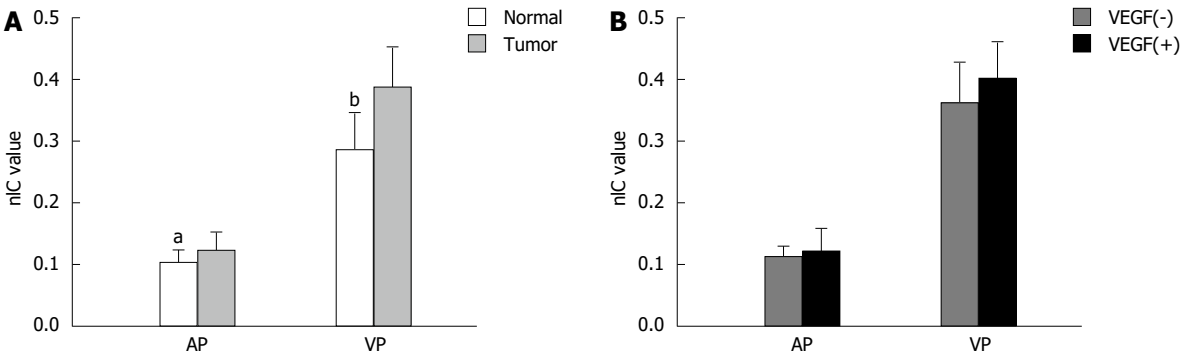

Figure 4 Comparison of normalized iodine concentration in arterial phase; and normalized iodine concentration in venous phase between the normal gastric wall and tumor site (A); comparison of normalized iodine concentration in arterial phase and normalized iodine concentration in venous phase between the vascular endothelial growth factor-positive and -negative group (B). [a]$P < 0.05$, [b]$P < 0.001$. VEGF: Vascular endothelial growth factor; nIC: Normalized iodine concentration; AP: Arterial phase; VP: Venous phase.

nIC-AP and nIC-VP showed statistically significant differences between differentiation categories ($P = 0.003$, $P = 0.001$, respectively) and between Lauren subtypes ($P = 0.016$, $P = 0.006$, respectively). The LSD test revealed that nIC-AP and nIC-VP were significantly different between intestinal and diffuse Lauren subtypes ($P = 0.005$, $P = 0.004$, respectively). nIC-VP was also significantly different between intestinal and mixed Lauren subtypes ($P = 0.013$), as shown in Table 4.

DISCUSSION

There were four major findings from this study. First, the bi-phasic nICs showed a significantly linear positive relationship with MVD in primary GC. The nIC-AP and nIC-VP correlated with MVD in different subgroups; the former correlated significantly with MVD in the relative earlier stage of advanced GC, while the latter correlated with MVD in the more advanced stage. Second, no significant correlation was found between nICs and VEGF. Third, nIC values in the normal gastric wall were observed to be significant lower than that in the tumor. Fourth, nICs were observed to differ between histological grades and Lauren subtypes of advanced GC; the greater the malignancy, the higher the nICs. Taken together, these observations demonstrate that quantification of iodine in spectral CT imaging have the potential to reflect angiogenesis of advanced GC.

Iodine, a commonly used CT contrast material, is generally known to produce higher attenuation at low tube voltage settings[18]. Based on this effect, spectral CT using high and low voltage switching settings could

Table 4 Difference of bi-phase normalized iodine concentration between different clinical-pathological subgroups

Varieties	*n*	nIC-AP		nIC-VP	
		mean ± SD	*P* value	mean ± SD	*P* value
Depth of invasion					
< T4	7	0.101 ± 0.042	0.195	0.363 ± 0.079	0.302
> T4	27	0.120 ± 0.031		0.392 ± 0.063	
Nodal status					
N0	11	0.112 ± 0.033	0.321	0.385 ± 0.085	0.923
N1-3	23	0.125 ± 0.035		0.387 ± 0.057	
Histologic differentiation					
Highly and moderately differentiated	17	0.100 ± 0.028	0.003[a]	0.352 ± 0.048	0.001[a]
Poorly differentiated	17	0.132 ± 0.031		0.421 ± 0.065	
Lauren subtype					
Intestinal type	15	0.099 ± 0.029	0.016[a]	0.347 ± 0.048	0.006[a]
Mixed type	7	0.120 ± 0.032		0.417 ± 0.061	0.013[1,a]
Diffuse type	12	0.135 ± 0.030	0.005[2,a]	0.417 ± 0.067	0.00[2,a]
Tumor location					
Cardia/Fundus	14	0.109 ± 0.033	0.445	0.385 ± 0.046	0.874
Gastricum	9	0.128 ± 0.024		0.387 ± 0.064	
Antrum	11	0.116 ± 0.040		0.387 ± 0.091	
Sex					
Male	23	0.113 ± 0.033	0.377	0.390 ± 0.069	0.633
Female	11	0.124 ± 0.035		0.378 ± 0.062	

[1]One-way ANOVA-LSD, mixed type *vs* intestinal type; [2]Diffuse type *vs* intestinal type. [a]$P < 0.05$. nIC-AP: Normalized iodine concentration in arterial phase; nIC-VP: Normalized iodine concentration in venous phase; MVD: Microvessel density; SD: Standard deviation.

differentiate materials of the same density[19] and the IC value could be extracted[20]. With water-iodine based material decomposition images, Lv *et al*[9] concluded that the IC in images of hepatic lesions acquired in a quantitative parameter was highly precise. Thieme *et al*[21] found a very good correlation between vessel occlusion depicted at CTA and IC defects in the dual-energy image, indicating that iodine distribution in the parenchyma is closely related to pulmonary perfusion. Therefore, the IC may be considered as an indirect marker of perfusion and tumor vascularity.

Tumor angiogenesis is defined as the formation of new blood vessels from pre-existing vessels[22]. MVD and identification of VEGF in tissues are commonly used biomarkers of angiogenesis[23]. In previous studies, the relationships between dynamic contrast-enhanced perfusion CT parameters and immunohistological markers of angiogenesis have been studied in different tumors, including colorectal cancer[24], advanced GC[6,25], lung cancer[26], *etc.* However, the studies produced discrepant results on whether the use of blood flow or permeability surface area product were efficacious.

Our results proved a positive linear relationship between IC and MVD in primary GC. The nIC values were significantly elevated in the tumor as compared to the normal gastric wall. Similar results were acquired by Pang *et al*[27], who considered the nIC value of the infarcted myocardium to be an important indicator of MVD in the 1-min and 3-min CT images. Additionally, the study by Hu *et al*[12] indicated that the nIC values of three-phase scans had a positive correlation with MVD for detecting the therapeutic response in a pancreatic carcinoma xenograft nude mouse model. Taken together, spectral CT imaging can be used to evaluate angiogenesis in disease.

On the other hand, nIC had a low correlation with VEGF expression, and no significant differences were found in the comparison between IC parameters and VEGF group. VEGF, one of the most prominent biomarkers of angiogenesis studied to date, has been shown to correlate well with CT perfusion in peripheral pulmonary nodules[28]. Moreover, the study of Zhou *et al*[13] in a rabbit VX2 liver model suggested that nIC and contrast-enhanced ultrasound parameters positively correlated with VEGF and FGF2 expression, while several reports failed to show such a correlation, for example, the GC study performed by Yao *et al*[6] and the colorectal cancer research by Goh *et al*[24]. The relationship between VEGF expression and imaging of tumor vascularity is complex. Further investigations of IC and VEGF in a large population with different clinical-pathology are needed.

At the same time, nIC in arterial phase and venous phase displayed different character when stratified. The nIC-AP showed correlations with MVD in relative earlier and differentiated advanced GC, while nIC-VP correlated with MVD in more advanced stage and poorly differentiated advanced GC.

In terms of the acquisition time of our routine abdominal CT scanning, the AP was performed at about 25-30 s after contrast media injection, when the mucosa at the lesion presented as a focal enhanced line. nIC-AP can reflect the blood supply and functional capillary density. In the VP of GC, the markedly increased interstitial fibrous tissue reduced the flow-out speed of the contrast media[29]; thus, more dysfunctional neo-vessels should be considered. Therefore, the nIC-VP may represent the distribution of iodine in

interstitial spaces. Under such conditions, the bi-phasic nIC demonstrated the vascular character of GC, and nIC-VP may be better correlated with MVD in advanced GC.

Furthermore, both nICs were different between histological differentiations and Lauren subgroups. The nIC-VP was more effective than nIC-AP in displaying the difference between Lauren subtypes. In general, the nIC value was higher in poorly differentiated and diffused types than that in highly or moderately differentiated and intestinal types. A previous study[30] has found that the degree of tumor angiogenesis was closely related to the pathological grade, that is, the poorer the differentiation of the tumor, the higher the MVD values. Another study[31] demonstrated a correlation between MVD and tumor histological type according to the Lauren classification. Accordingly, it is likely that the nIC value can be used to evaluate histology by mapping the neovascularization of advanced GC. The finding in the present study was also supported by the results of Pan *et al*[15] and Wang *et al*[32] in GC research.

The nICs increased in patients with serosal involvement and lymphatic metastasis, but the differences from patients without serosal involvement and lymphatic metastasis did not reach significant difference, for reasons that are not immediately clear, as these clinical-pathological features should reflect the functional status of the vasculature. Both nIC-AP and nIC-VP correlated with MVD of GC located in the antrum and occurring in different genders. However, nICs were not very effective in differentiating between these groups.

There are several potential limitations in our study. First, the tumor vasculature is spatially heterogeneous. Although we selected specimens carefully under the guide of two major specialists, the excision level was barely achieved. It may be questioned whether the part of the histopathological part selected and matched to the imaging measurement represented the angiogenesis of the whole tumor. Second, the sample numbers were limited, especially when subdivided by clinical classification. A larger prospective investigation is needed to confirm the present findings. Third, given the limitations of CT resolution, flat and light ulcerous lesions without enhancement would have been missed in the images, which inducing selection bias.

In conclusion, the bi-phasic nIC values had a positive linear correlation with MVD. nIC-AP reflected the angiogenesis in relatively earlier and well-differentiated advanced GC, while nIC-VP reflected this in further advanced and poorly differentiated GC. Spectral CT with quantitative IC value offers a new choice to evaluate the angiogenesis of gastric cancer noninvasively.

COMMENTS

Background

Angiogenesis is fundamental to the growth, invasion, and metastasis of gastric cancer (GC). To date, the standard method for studying angiogenesis has been histopathological counting of micro-vessel density (MVD). This is impractical for patients who undergoing anti-angiogenesis or chemotherapy. The current trial was designed to evaluate if the iodine concentration (IC) generated by spectral computed tomography could non-invasively judge the features of tumoral MVD and vascular endothelial growth factor with clinical data.

Research frontiers

Iodine-water material-decomposition (MD) images can quantitatively map the IC of the tissue in enhanced scanning. In this study, there is suggestion that IC value has the potential to reflect angiogenesis of advanced GC.

Innovations and breakthroughs

The authors measured the normalized IC value in spectral computed tomography (CT) with manually drawn regions of interests to avoid the selection bias. Data stratified by clinical subgroups further revealed the connection between imaging index and patho-index. They finally proved that the normalized IC value in different scanning phase could reflect angiogenesis in different pathological subgroups of advanced GC.

Applications

Radiological-pathological correlation in angiogenesis, although with not much high coefficient, will offer a new choice to clinical decision in judging the status of GC, especially for neoadjuvant chemotherapy or radiochemotherapy patients.

Terminology

IC: Iodine is a commonly used contrast material in performing enhanced CT scanning. The iodine concentration here refers an imaging data, representing iodine distribution of the organ with spectral CT modality, not the real iodine concentration of contrast material itself.

Peer-review

The study on the spectral computed tomography in advanced gastric cancer is quite interesting with novelties.

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EVIDENCE-BASED MEDICINE

Inflammatory bowel disease: An evaluation of health information on the internet

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Abstract

AIM

To evaluate the quality and accuracy of websites written to the public on inflammatory bowel disease (IBD) (Crohn's disease and ulcerative colitis) and assess their readability level.

METHODS

Google™, Bing™, and Yahoo™ search engines were searched independently by three researchers in December 2014. Only English-language websites were selected on the basis of predetermined inclusion and exclusion criteria. Researchers independently evaluated the quality of each website by using the DISCERN and the HONcode instruments. The readability levels were calculated using two formulas; the Flesch-Kincaid Grade Level Index, and the Coleman-Liau Readability Index. The agreement between the evaluators was calculated using Cohen kappa coefficient.

RESULTS

Eighty-four websites were finally identified. Scores varied from a minimum DISCERN score of 18 to a maximum of 68 [mean ± SD, 42.2 ± 10.7; median = 41.5, interquartile range, interquartile range (IQR) = 15.8] and a minimum score of HONcode of 0.14 and a maximum of 0.95 (mean ± SD, 0.16 ± 0.19; median = 0.45, IQR = 0.29). Most of these websites were reviewed in 2014 and 2015 (n = 51). The creators of these websites were: universities and research centers (n = 25, 30%), foundations and associations (n = 15, 18%), commercial and pharmaceutical companies (n =

25, 30%), charities and volunteer work (*n* = 9, 10%), and non-university educational bodies (*n* = 10, 12%). The Flesch-Kincaid Grade Level readability score (mean ± SD) was 11.9 ± 2.4 and the Coleman-Liau Readability Index score was 12.6 ± 1.5. Significant correlation was found between the two readability scores (R^2 = 0.509, *P* = 0.001). The overall agreement between evaluators measured by Cohen kappa coefficient was in the range of 0.804-0.876; rated as "Good".

CONCLUSION

The DISCERN and the HONcode scores of websites varied and the readability levels of most websites were above the public readability level. The study highlights the areas that need further improvement and development in patient education online materials about IBD.

Key words: Inflammatory bowel disease; The internet; Patients' information; Evidence; Patients' education; Online resources

Core tip: This is a comprehensive study analyzing the quality and accuracy of content and the readability level of websites in the English language on inflammatory bowel disease dedicated to the public. Two standardized instruments were used in assessing quality and accuracy and two methods were used in calculating readability level. The study showed variability in scores and the readability levels of most websites were above that for the public. Based on evidence, the study highlights the need for improving online patient education.

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INTRODUCTION

Inflammatory bowel disease (IBD) refers to two chronic inflammatory disorders, Crohn's disease and ulcerative colitis. Both are life long, relapsing disorders of unknown etiology; possibly the result of interaction between genetic and environmental factors[1]. The diagnosis of these disorders is based on clinical features, endoscopy, and histological changes[2]. Crohn's disease may affect any part of the gastrointestinal tract but most commonly affects the distal ileum and proximal colon. The disease is characterized by inflammatory changes involving all the layers of the affected regions. In contrast, ulcerative colitis is characterized by continuous ulceration starting in the rectum and limited to the colonic mucosa[3]. IBD occurs worldwide with the highest incidence in developed countries mainly North America, United Kingdom and northern Europe. The incidence of ulcerative colitis in North America is approximately 19.5 per 100000 person years and 243 per 100000 person years in Europe while the incidence of Crohn's disease in North America is approximately 20.2 per 100000 person years and 12.7 per 100000 person years in Europe[4]. The aims of treatment are to induce remission in active disease and to maintain remission/prevent relapse. Therapeutic modalities include lifestyle modification, nutritional support, and medications. Surgery is reserved for the treatment of complications or when the medical therapy is ineffective. In addition to other complications, patients with IBD are at a higher risk of developing colorectal cancer. Therefore, patients have to undergo to regular checkup for early detection of the development of colon cancer[5].

With this information in mind, patients with IBD, as it is the case with other chronic diseases, usually seek information about the nature of the disease, its causes, investigations needed to diagnose the disease and therapeutic options. The advances in treatment modalities and options, and the increasing desire for patients to participate in decision-making about treatment choices necessitate the need for resources to support these decisions. Nowadays, patients have increasingly used the Internet as a source of health information because of its global accessibility, speed, and cost effectiveness[6]. Approximately 80% of the Internet users look for medical or health-related information through the Internet[7]. The topics most searched were information about specific disease or medical condition, treatment options, diet and nutrition, exercise and fitness and medications[7]. The increasing use of the Internet embraces a variety of aspects of topics searched, which gives the person an opportunity to investigate their questions from several resources. However, with the abundance of such information there is concern about the quality, accuracy, and readability level of the information available on the Internet about health care[8].

Therefore, the aims of this study were: to evaluate the quality, and accuracy of web-based information about IBD using two instruments, the DISCERN and the HONcode, as well as calculate the readability level by using two formulas, the Flesch-Kincaid Grade Level Index, and the Coleman-Liau Readability Index. The rationales for the study were to assess the educational usefulness of web-based information on IBD particularly their quality, accuracy and areas of deficiencies that need improvement. Also to assess whether these resources are easily read and understood by the public. Therefore our research questions are: (1) for the websites targeting the public and patients with IBD, what is the accuracy and the quality of these information resources? and (2) does the readability level of these online resources match with the recommended level for the public?

MATERIALS AND METHODS

Search design

In this study we assessed websites written for patients and the public on IBD by searching three search engines (Google™, Bing™ and Yahoo™), the selection of these three search engines was based on current statistical information that showed that these engines are the most searched by the public for health information[9]. The quality and accuracy of information provided on websites were assessed using two instruments: the DISCERN (www.discern.org.uk) and HONcode (www.hon.ch/HONcode/) instrument. Details about these instruments and the justification for selecting them are discussed later. The readability of the websites was assessed using two methods: the Flesch-Kincaid Grade Readability Level and the Coleman-Liau Readability Index. After piloting the work and ensuring satisfactory use of these instruments by researchers, the work was carried out to assess the quality of websites. The Institutional Review Board, College of Medicine King Saud University, has approved the project and the approval number: F06/2014.

Searching the internet

Using the following key words: "inflammatory bowel disease", "Crohn's disease", "ulcerative colitis", "inflammatory bowel disease patient information", "Crohn's disease patient information", and "ulcerative colitis patient information", three search engines (Google™, Bing™ and Yahoo™) were searched. Researchers independently from 1 to 20 December 2014 conducted the search. Information for each website was recorded; these included: website title, website URL, name of creator, year of publication on the Internet, last date updated, and the objectives of the website. This information about each website was collected using the following online meter: http://whois.domaintools.com/. The data collected were evaluated on the bases of the inclusion and the exclusion criteria.

Inclusion and exclusion criteria

The inclusion criteria included: (1) websites covering public education about Crohn's disease, ulcerative colitis or IBD; and (2) websites focusing on patient education and in the English language. The excision criteria comprised: (1) websites addressing doctors or health professionals; (2) lectures, and advertisement on IBD; (3) websites in languages other than English; and (4) presentations at conferences.

Assessing accuracy and quality of information

Two instruments were used in assessing the quality and accuracy of information provided, namely the DISCERN instrument and the HONcode instrument. These two instruments have been widely used in the literature in assessing information on the Internet particularly health related issues and patients' education online resources[8,10-12]. More details about these two instruments can be summarized as follows:

DISCERN instrument: This instrument is a standardized set of criteria for judging the quality of health information and is written for the public to assess treatment options[8,10-12]. The DISCERN instrument was created by the University of Oxford, and the project was funded by the British Library and the National Health Service (NHS) Research & Development Programme[13] The instrument consists of 15 questions plus an overall quality rating question. The questions can be grouped under the three key topics as follows: Questions 1 to 8 addressing reliability, Questions 9 to 15 addressing specific detail about the information provided and treatment choices, and Q16 covering the overall quality rating[14]. The instrument has been used to assess healthcare-related websites and online resources. For example, the quality of patients' information on surgical treatment of haemorrhoids[12], and colorectal cancer information[11].

HONcode instrument: The Health on the Net (HON) Foundation, a non-profit, and non-government organization created this instrument in 1995. The instrument focuses on key questions on the provision of health information available on the Net, and provides a code of conduct addressing eight principles: (1) authoritative (indicates the qualifications of the authors); (2) complementarity (Information should support, not replace, the doctor-patient relationship); (3) privacy (Respect the privacy and confidentiality of personal data submitted to the site by the visitor); (4) attribution [cite the source(s) of published information, date medical and health pages]; (5) justifiability (site must back up claims relating to benefits and performance); (6) transparency (Accessible presentation, accurate email contact); (7) financial disclosure (Identify funding sources); and (8) advertising policy (Clearly distinguish advertising from editorial content)[15-17]. To earn HONcode certification, a website must conform to the eight principles of the HONcode of Conduct. An HONcode expert then assesses the candidate website using precise guidelines for each principle. Recently, the HON Foundation has developed an automated system to assist in detecting a website's HONcode conformity. Therefore, the automated assistance in conducting HONcode reviews can expedite the current time-consuming tasks of HONcode certification and ongoing surveillance. A recent study showed that there is concordance between automated and expert manual compliance detection for the criteria[18]. In this research we have used the electronic system available at: http://www.readabilityformulas.com/free-readability-formula-tests.php.

The HONcode has been widely used in the literature in assessing health-related websites[19]. The two instruments, the DISCERN and HONcode, do not exactly cover the same issues/topics, although there are some

overlaps. Therefore, using these two instruments with these differences in mind could provide a better evaluation of the websites.

Piloting the study

The aims of piloting the study were: (1) to introduce the two instruments to the researchers and orient them on how to use each instrument in assessing the websites; and (2) identify difficulties facing the researchers on applying the two instruments and the sources of disagreements among them. Such exercise prior to the implementation of the two instruments was vital for ensuring optimal use of the instruments and maximizing the degree of agreement among evaluators when they apply these two instruments in the actual research. The piloting part was conducted as follows: (1) approximately 10 websites other than those identified for the research study were evaluated independently by three researchers using the two instruments; (2) the results of their evaluation were discussed with the aim to identify sources for difficulties/disagreements; (3) the identified differences were resolved after discussing them reaching to a solution; and (4) the same process was repeated on another 10 websites until the agreement between the researchers reached to an optimal level[20].

Conduction of the study

Along with the same approach described under piloting the study, the researchers evaluated the websites identified by applying the two instruments on each website and giving a score. The process was conducted by each researcher independently first by applying the DISCERN instrument then the HONcode instrument. The results of the assessment were placed on an Excel sheet for each researcher. The degree of agreement was measured using Cohen kappa coefficient[21].

Calculating website readability

The aims of calculating readability level of websites was to assess if they were written at the readability level of the general public and patients; should not exceed the 6th grade readability level[22,23]. Two methods were used to calculate readability: The Flesch-Kincaid Grade Level Index[24], and the Coleman-Liau Readability Index[25,26]. It was decided to use these two methods rather than one method so that we can compare the readability scores and examine if there were an agreement between the two methods, and hence strengthening the outcomes of our readability assessment and our conclusions. The two methods can be summarized as follows:

Flesch-Kincaid grade level index: This test helps in indicating how difficult a reading passage in the English language to understand. The test was developed by Rudolf Flesch and finalized by J Peter Kincaid for use by the United States Navy, hence the name of the test[24]. The test is based on the word length and the sentence length and is based on the following formula:

0.39 × [(total words)/(total sentences)] + 11.8 × [(total syllables)/(total words)] - 15.59

This method has been widely used in assessing the readability of websites and educational material[27].

Coleman-Liau readability index: This test differs from the above method in relying on characters instead of syllables per word. It enables the users however to grade the readability level. It has been widely used in assessing the readability of educational material[27].

We used a free online calculator (www.readability-formulas.com) to calculate the readability level using the two readability methods. As per instructions provided by the website, the top, middle and bottom 150-200 words of each website were placed in the calculator and then the text readability was checked by calculating the number of sentences, words, syllables, and characters in the sample. A sufficient sample size of four to five full sentences; approximately 200-500 words in total were used. The scores recorded for each website were placed on an Excel sheet and reviewed by two other researchers before conducting final analysis for the means and standard deviations.

Grouping the websites under five categories

Assessment of the identified websites revealed variability in their creators. These can be grouped into 5 categories: (1) university, affiliated hospitals, and research centres; (2) foundations and associations; (3) commercial and pharmaceutical companies; (4) charities and volunteer works; and (5) non-university educational bodies such as colleges, academies, and councils. The grouping of websites under these five categories was carried out by researchers independently and was reviewed in a meeting for any disagreements.

Statistical analysis

The collected data were placed on an Excel Sheet (Microsoft Excel for Mac 2011, Microsoft Corporation, Redmond, WA, United States). All analysis was conducted by using SPSS software (SPSS Statistics version 22 for Mac, IBM Corporation, Armonk, NY, United States). For the data collected from measuring website accuracy, and the readability scores, the means, standard deviations, the median and interquartile range (IQR) were calculated. Pearson correlation studies and *P*-values for significance were calculated to examine if there were correlations between the scores obtained from the two readability methods[28]. A *P*-value of < 0.05 was considered significant. The agreement between the evaluators measured by the degree of inter-rater agreement using Cohen kappa coefficient was also carried out using SPSS software. This has been interpreted as "Poor", if the results in the range:

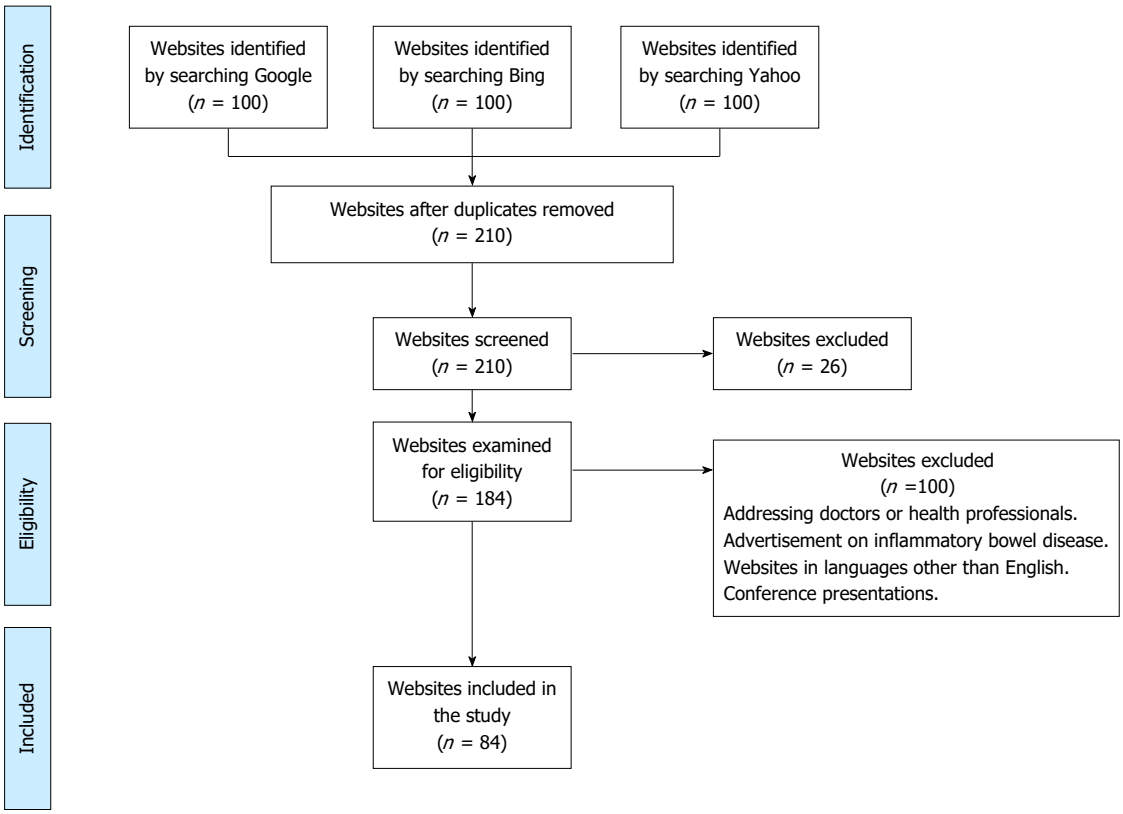

Figure 1 PRISMA flowchart showing the websites on inflammatory bowel disease searched on the Internet and those finally included in the study.

0.21-0.40; "Fair" 0.41-0.60; "Moderate" 0.61-0.80; "Good" 0.81-1.00.

RESULTS

General information about websites

The search of the three databases, Google™, Bing™ and Yahoo™, resulted in the identification of 300 websites. After the duplicates were removed we ended with 210 websites. On applying the inclusion and exclusion criteria, 84 websites were finally identified and included in the study (Figure 1).

Table 1 summarizes the general information about the 84 websites, including: website title, URL, author/ownership, year created, last updated, number of pages, number of tables, images and illustrations. The oldest two websites were created by the University of North Carolina (UNC), School of Medicine, North Carolina, United States and the Department of Surgery, University of California, California, United States, while the most recent was published in 2013 and created by New Health Guide, United States.

For other websites, four websites were published in 1987-1994, 44 were published in 1995-2002, and 29 were published in the years 2003-2011. Only four websites were difficult to identify the exact year of their publication. Websites were updated regularly, 51 websites were updated in 2015 and 2014, while 33 websites were updated earlier, including one website was updated in 2006.

Of the 84 websites, 60 websites comprised 1-5 pages, 16 websites had 6-10 pages, 8 had more than 11 pages. The website titled Crohn's disease by the University of Maryland medical center had the highest number of pages, 20 pages. The number of tables varied from zero to 6. Out of the 84 websites only 24 websites used tables to explain their content. The total number of tables in these websites was 55. The number of images varied from zero to 27. Out of the 84 websites only 42 websites had images to explain the content. The total number of images in these websites was 141. Again the number of illustrations varied from zero to 10. Out of the 84 websites, only 28 had illustrations to explain the content. The total number of illustrations was 53.

DISCERN and the HONcode scores of websites

In order to calculate the accuracy of the websites, we used two instruments, the DISCERN and the HONcode instruments. Table 2 summarizes the scores calculated from applying the DISCERN and the HONcode scores expressed as mean ± SD for each website. The DISCERN scores varied from a minimum of 18 to a maximum of 68 (mean ± SD, 42.2 ± 10.7; median = 41.5, IQR = 15.8). The lowest DISCERN score was scored by the website, Crohn's Disease Diagnosis, New health guide, while the highest DISCERN score was scored by the website, Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases. The HONcode trust worthy scores

Table 1 Summarizes general information about websites on inflammatory bowel disease included in the study

No.	Website title, Organisation	URL	Authority/ownership, state, country	Year created	Last updated	Number of pages	Number of tables	Number of images	Number of illustrations
1	Inflammatory Bowel Disease (IBD), Mayo Clinic	http://www.mayoclinic.org/diseases-conditions/inflammatory-bowel-disease/basics/definition/con-20034908	Mayo Foundation for Medical Education and Research, Arizona, United States	1997	04 Feb 2014	11	0	0	0
2	Inflammatory Bowel Disease Health Center, WebMd	http://www.webmd.com/ibd-crohns-disease/	WebMD, Inc., Georgia, United States.	1998	06 Sep 2013	2	0	3	0
3	Inflammatory Bowel Disease (IBD), Center for Disease Control and Prevention (CDC)	http://www.cdc.gov/ibd/	Centers for Disease Control and Prevention (CDC), Georgia, United States.	1999	04 Sep 2014	3	0	1	1
4	Inflammatory Bowel Disease, NHS Choices	http://www.nhs.uk/conditions/inflammatory-bowel-disease/pages/introduction.aspx	NHS England, Wakefield, United Kingdom.	1996	29 Apr 2013	2	0	0	0
5	What are Crohn's and Colitis? Crohn's and Colitis Foundation	http://www.ccfa.org/what-are-crohns-and-colitis/	Crohns and Colitis Foundation of America, New York, United States.	1996	14 Apr 2014	1	0	1	0
6	Inflammatory Bowel Disease, KidsHealth	http://kidshealth.org/parent/medical/digestive/ibd.html	Kids Health Organisaion, The Nemours Foundation, Orlando, United States.	1995	27 Jan 2015	4	0	1	0
7	Inflammatory Bowel Disease (IBD). FamilyDoctor	http://familydoctor.org/familydoctor/en/diseases-conditions/inflammatory-bowel-disease.html	American Academy of Family Physicians, New Jersey, United States.	1998	22 Jan 2015	7	0	0	0
8	Inflammatory Bowel Disease, Healthline	http://www.healthline.com/health/inflammatory-bowel-disease#Overview1	Healthline, California, United States.	2004	29 Nov 2011	6	0	0	0
9	Crohn's and Colitis, Australia	https://www.crohnsandcolitis.com.au/about-crohns-colitis/inflammatory-bowel-disease/	Crohn's and Colitis Australia, Victoria, Australia.	2009	24 Jul 2014	3	0	1	0
10	Crohn's and Colitis UK	http://www.crohnsandcolitis.org.uk/information-and-support/information-about-ibd/what-is-IBD	Crohn's and Colitis UK, United Kingdom.	2010	13 Nov 2014	2	0	1	0
11	Inflammatory Bowel Disease (IBD) (Intestinal Problems of IBD), MedicineNet	http://www.medicinenet.com/inflammatory_bowel_disease_intestinal_problems/article.htm	Medicine.Net.com, WebMed Network, New York, United States.	1995	6 Sep 2013	12	0	27	0
12	Inflammatory Bowel Disease Center, Cedars-Sinai	http://www.cedars-sinai.edu/Patients/Programs-and-Services/Inflammatory-Bowel-Disease-Center/	Cedars-Sinai Medical Center, California, United States.	1992	5 Jul 2013	2	0	0	0
13	Inflammatory Bowel Diseases Symptoms and Treatment: Livescience	http://www.livescience.com/39880-inflammatory-bowel-disease.html	Tanya Lewis, LiveScience Contributor, New York, United States.	2001	10 Apr 2014	11	0	1	0
14	Inflammatory Bowel Diseases Program, Penn Medicine.org	http://www.pennmedicine.org/gastroenterology/patient-care/gi-diseases/inflammatory-bowel-disease-ibd/	Penn Medicine, Pennsylvania, United Stats.	2003	6 Nov 2014	4	0	0	0
15	Inflammatory Bowel Diseases Support Groups, IBDsupport.org	http://www.ibdsupport.org/	IBD support.org, Utah, United States.	2011	30 May 2014	2	0	0	0
16	Inflammatory Bowel Disease (IBD), ABC Health and wellbeing	http://www.abc.net.au/health/library/stories/2012/02/22/3435688.htm	Australian Broadcasting Corporation, NSW, Australia.	2001	3 Dec 2014	4	0	1	0

17	Inflammatory Bowel Disease (IBD), GIKids	http://www.gikids.org/content/7/en/IBD	GIKids and The NASPGHAN Foundation, Pennsylvania, United States.	2009	11 Jun 2013	2	0	1	0
18	Inflammatory Bowel Disease, Vitamin D Council	https://www.vitamindcouncil.org/health-conditions/inflammatory-bowel-disease/	The Vitamin D Council, California, United States.	2007	17 Jun 2011	6	0	0	0
19	Inflammatory Bowel Disease Symptoms and Diagnosis, Seattle children's	http://www.seattlechildrens.org/medical-conditions/digestive-gastrointestinal-conditions/ibd-symptoms/	Children's Hospital and Regional Med. Ctr, Washington, United States.	1993	23 Apr 2014	2	0	0	0
20	Crohn's Disease, Patient.co.uk	http://www.patient.co.uk/health/crohns-disease-leaflet	Patient, Patient information Publications, Leeds, United Kingdom.	1997	05 Mar 2013	8	0	0	1
21	Patient Information Crohn Disease (Beyond and the Basics), Uptodate	http://www.uptodate.com/contents/crohn-disease-beyond-the-basics	UpToDate, Wolters Kluwer Health, Illinois, United States.	1998	29 Jul 2014	5	0	0	0
22	Crohn's Disease, Centre for digestive diseases	http://www.cdd.com.au/pages/disease_info/crohns_disease.html	The Centre for Digestive Diseases, NSW, Australia.		08 Jul 2013	4	0	0	0
23	Crohn's Disease, Patients: British Society for Gastroenterology	http://www.bsg.org.uk/patients/general/crohn-s-disease.html	British Society of Gastroenterology, London, United Kingdom.	1996	05 Aug 2014	6	0	0	0
24	Crohn's Disease, Bupa	http://www.bupa.co.uk/health-information/directory/c/crohns-disease	The British United Provident Association Ltd, London, United Kingdom	1996	16 Jun 2014	6	0	0	1
25	Crohn's Disease, University of Maryland Medical Center	http://umm.edu/health/medical/reports/articles/crohns-disease	University of Maryland Medical Center, Maryland, United States	1996	19 Sep 2013	20	0	0	0
26	Crohn's Disease, Symptoms, Diagnosis, Treatment, Southern Cross	https://www.southerncross.co.nz/AboutTheGroup/HealthResources/MedicalLibrary/tabid/178/vw/1/ItemID/523/Crohns-disease-symptoms-diagnosis-treatment.aspx	Southern Cross Healthcare Group; Auckland, New Zealand.	1998	01 Feb 2015	5	0	0	0
27	Crohn's Disease, American family physician	http://www.aafp.org/afp/2011/1215/p1379.html	American Academy of Family Physicians, Kansas, United States	1995	02 Jul 2014	2	0	0	0
28	What is Crohn's Disease? What Causes Crohn's Disease? MNT	http://www.medicalnewstoday.com/articles/151620.php	Christian Nordquist, MNT, Sussex, United Kingdom.	2003	02 Jan 2014	7	0	0	0
29	Crohn's Disease, Netdoctor	http://www.netdoctor.co.uk/diseases/facts/crohnsdisease.htm	NetDoctor.co. Ltd, London, United Kingdom.	1998	24 Aug 2014	7	0	1	0
30	Crohn's Disease, UCSF medical center	http://www.ucsfhealth.org/conditions/crohns_disease/	University of California San Francisco Medical Center, California, United States.	2000	04 Jan 2012	2	0	0	0
31	Crohn's Disease Symptoms and Treatment, US.news Wellness	http://health.usnews.com/health-news/health-wellness/articles/2013/08/03/crohns-disease-symptoms-and-treatment	Guido Zanni, US News, New York, United States,	1995	22 Jan 2015	3	0	0	0
32	What are the treatments for Crohn's disease? Beth Israel Deaconess Medical Center	http://www.bidmc.org/Centers-and-Departments/Departments/Digestive-Disease-Center/Inflammatory-Bowel-Disease-Program/Crohns-Disease/What-are-the-treatments-for-Crohns-disease.aspx	Beth Israel Deaconess Medical Center, Massachusetts, United States.	2002	16 Mar 2006	15	0	4	1

33	Diagnosing Crohn's, Crohn's and Me.	http://www.crohnsandme.com/crohns-information/crohns-disease-diagnosis.aspx	UCB Multinational Biopharmaceutical Company, Brussels, Belgium	2005	29 Apr 2014	9	1	1	1
34	Understanding Crohn's Disease, Crohn's and Colitis.	http://www.crohnsandcolitisinfo.com/Crohns/What-is-Crohns-Disease	Crohn's and Colitis, Illinois, United States	2011	02 Oct 2014	8	0	1	1
35	Learning About Crohn's Disease, National Human Genome Research Institute	http://www.genome.gov/25521854	National Human Genome Research Institute, Massachusetts, United States		27 Sep 2011	2	0	0	0
36	Crohn's Disease, UPMC Life Changing Medicine	http://www.upmc.com/services/digestive-disorders-center/services/ibd/conditions/pages/crohns-disease.aspx	UPMC Digestive Disorders Center, UPMC Presbyterian, Pennsylvania, United States	1999	04 Mar 2014	3	0	0	0
37	Crohn's Disease, Cincinnati Children's	http://www.cincinnatichildrens.org/health/c/crohns/	Cincinnati Children's Hospital Medical Center, Ohio, United States	1998	15 may 2012	4	0	1	0
38	Crohn's Disease, Cleveland clinic	http://my.clevelandclinic.org/health/diseases_conditions/hic_Inflammatory_Bowel_Disease_IBD_QandA/hic_Crohns_Disease	The Cleveland Clinic Foundation, Ohio, United States	1998	02 Jan 2015	2	0	0	0
39	Treatment of Crohn's Disease, UNC Multidisciplinary Center for IBD Research and Treatment	http://www.med.unc.edu/gi/specialties/ibd/about-ibd/treatment-of-ibd-1/treatment-of-crohns-disease	UNC, School of Medicine, North Carolina, United States	1986	07 Mar 2013	9	0	9	10
40	Crohn's Disease, Emedicine health	http://www.emedicinehealth.com/crohn_disease/article_em.htm	EMedicine.com Inc, WebMD Network, Georgia, United States	2003	06 Sep 2013	10	0	0	1
41	Understanding Crohn's Disease and Ulcerative Colitis. Australian Gastroenterology Institute.	http://www.nevdgp.org.au/info/gastro/crohns.htm	Australian Gastroenterology Institute, Digestive Health Foundation, New South Wales, Australia		02 Jun 2014	3	0	0	0
42	Crohn's Disease, Health Centers	http://www.drweil.com/drw/u/ART00339/Crohns-Disease.html	Weil's Foundation, Arizona, United States	1999	16 Jan 2012	3	0	1	0
43	Crohn's Disease-An Overview, the Royal Children's Hospital Melbourne	http://www.rch.org.au/kidsinfo/fact_sheets/Crohns_Disease_an_overview/	The Royal Children's Hospital Melbourne, Victoria, Australia		19 Jun 2014	3	0	0	0
44	Fighting Inflammatory Bowel Disease Together, the Irish Society for Colitis and Crohn's Disease	http://www.iscc.ie/page.php?id=18&title=What%20is%20IBD	The Irish Society for Colitis and Crohn's Disease, Dublin, United Kingdom	2000	05 Jan 2014	4	0	10	1
45	Crohn's Disease Diagnosis, New health guide	http://www.newhealthguide.org/Crohn%27s-Disease-Diagnosis.html	New Health Guide, United States	2013	10 Feb 15.	3	0	1	0
46	Crohn's Disease, HealthDay.	http://consumer.healthday.com/encyclopedia/digestive-health-14/digestion-health-news-200/crohn-s-disease-644392.html	HealthDay, New York, United States	2002	15 Apr 2014	4	0	0	0
47	Ulcerative Colitis, Wikipedia	http://en.wikipedia.org/wiki/Ulcerative_colitis	Wikimedia Foundation, Inc, California, United States	2001	08 May 2012	14	6	6	1
48	Living with UC, Do You Know Your Treatment Options?	http://www.livingwithuc.ca/	Janssen Inc., Canada	2010,	May 2014	8	4	0	2

49	What Is Ulcerative Colitis? Everyday Health	http://www.everydayhealth.com/conditions/ulcerative-colitis	Everyday Health Media, LLC, New York, United States	2004	10 Feb 2014	14	0	0	0
50	What Is Ulcerative Colitis? News Medical	http://www.news-medical.net/health/What-is-Ulcerative-Colitis.aspx	The AZO Network, New South Wales, Australia	2004	26 Feb 2015	2	0	0	0
51	Crohn's Disease and Ulcerative Colitis, Better Health Channel	http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Crohn%27s_disease_and_ulcerative_colitis	Crohn's and Colitis, The State Government of Victoria, Victoria, Australia	2007	28 Sep 2014	4	0	0	0
52	Ulcerative Colitis, Jackson Siegelbaum Gastroenterology	http://gicare.com/diseases/ulcerative-colitis/	Jackson Gastroenterology Ltd, Central Pennsylvania, Pennsylvania, United States	1997	07 April 2014	3	0	0	3
53	Ulcerative Colitis, Crohn's and Colitis Canada	http://www.crohnsandcolitis.ca/site/c.dtJRL9NUJmL4H/b.9012449/k.C223/Ulcerative_Colitis.htm	Crohn's and Colitis, Canada	2008	19 Dec 2013	12	0	0	1
54	Ulcerative Colitis, Healthgrades	http://www.healthgrades.com/conditions/ulcerative-colitis	HealthGrades, Inc, Colorado, United States	1999	08 May 2014	4	0	0	0
55	Information for Those with Ulcerative Colitis, Colitis UK	http://www.ulcerativecolitis.org.uk/	Colitis UK, Buckinghamshire, United Kingdom	2005	20 Oct 2013	10	0	0	0
56	Colitis and Chronic Ulcerative Colitis, Virginia Mason	https://www.virginiamason.org/ColitisandChronicUlcerativeColitis	Virginia Mason Medical Center, Washington, United States	1998	07 May 2008	3	0	0	0
57	Ulcerative Colitis, GastroNet	http://www.gastro.net.au/diseases/ulcerativecolitis.html	GastroNet Australia Pty Ltd, Canberra, Australia	2009	12 May 2014	5	2	4	5
58	Ulcerative Colitis, Ulcerative Colitis Net.	http://www.ulcerativecolitis.net/	Serovera, Florida, United States	2000	28 Feb 2014	9	3	1	0
59	Inflammatory Bowel Disease, Lab Tests Online	http://labtestsonline.org/understanding/conditions/inflammatory-bowel	American Association for Clinical Chemistry (AACC), Washington, DC, United States	2001	08 Nov 2010	3	3	0	2
60	Inflammatory Bowel Disease Fact Sheet, Womenshealth.gov	http://www.womenshealth.gov/publications/our-publications/fact-sheet/inflammatory-bowel-disease.html	Womenshealth.gov, the US Department of Health and Human Services	1995	29 Nov 2014	3	0	0	3
61	Inflammatory Bowel Disease (IBD), Innerbody	http://www.innerbody.com/diseases-conditions/ibd	InnerBody, California, United States	1996	02 Oct 2012	5	2	3	4
62	Inflammatory Bowel Disease (IBD), Rightdiagnosis	http://www.rightdiagnosis.com/i/inflammatory_bowel_disease/intro.htm	Rightdiagnosis. com, United States	2005	11 Jul 2013	2	1	5	0
63	Inflammatory Bowel Disease, Lifescript.com	http://www.lifescript.com/health/centers/digestive/related_conditions/inflammatory_bowel_disease.aspx	LifeScript, California, United States	1999	05 May 2014	1	5	2	1
64	Inflammatory Bowel Disease (IBD), MUSC Health	http://www.ddc.musc.edu/public/symptomsDiseases/diseases/smallBowel/IBD.html	Digestive Disease Center, The Medical University of South Carolina, South Carolina, United States	1990	21 May 2013	1	1	1	0
65	Inflammatory Bowel Disease (IBD): Ulcerative Colitis, Crohn's Disease, New York-Presbyterian Digestive Diseases.	http://nyp.org/services/digestive/ibd.html	NewYork-Presbyterian Hospital, New York, United States	1998	24 Aug 2007	1	2	0	0

66	Inflammatory Bowel Disease, Human Diseases and Conditions Forum.	http://www.humanillnesses.com/original/Her-Kid/Inflammatory-Bowel-Disease.html	Human Diseases and Conditions Forum, United States	2006	16 Oct 2013	2	3	1	0
67	Facts About Crohn's Disease, US. Food and Drug Administration	http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm107358.htm	US Food and Drug Administration, The US Department of Health and Human Services, Maryland, United States	1997	14 Oct 2014	1	3	2	1
68	Crohn's Disease Symptoms and warning Signs, SymptomFind	http://www.symptomfind.com/diseases-conditions/crohns-disease-symptoms-warning-signs/	Symptom.Find.Com. United States	2008	21 Jun 2014	3	2	1	2
69	Crohn's Disease-At a Glance, SixPartsWater.Org	http://www.sixpartswater.org/knowledge-centre/crohns-disease/glance	SixPartsWater.Org, United Kingdom	2007	02 Oct 2012	2	1	3	0
70	Ulcerative Colitis, eMedTV	http://colitis.emedtv.com/ulcerative-colitis/ulcerative-colitis.html	eMedTV, Washington, United States	2005	07 May 2014	3	0	5	2
71	Crohn's Disease, Department of Surgery, University of California.	http://colorectal.surgery.ucsf.edu/conditions--procedures/crohns-disease.aspx	Department of Surgery, University of California, California, United States	1986	10 Oct 2013	1	1	4	1
72	Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases	http://www.niddk.nih.gov/health-information/health-topics/digestive-diseases/crohns-disease/Pages/facts.aspx	The National Institutes of Diabetes and Digestive and Kidney Diseases, NIDDK, Maryland, United States	2002	20 Jul 2014	1	1	2	0
73	Crohn's Disease, Patient Education Center	http://www.patienteducationcenter.org/articles/crohns-disease/	Patient Education Center, Harvard Medical School, Harvard Medical Publications, Massachusetts, United States	2003	20 Jun 2014	2	0	3	0
74	Crohn's Disease Information, alot health	http://health.alot.com/conditions/crohns-disease-information--163	Alot Health.Com, Arkansas, United States	1994	15 Aug 2014	3	1	1	1
75	Crohn's Disease, Diagnose-me.Com	http://www.diagnose-me.com/symptoms-of/crohns-disease.html	Diagnose-me.Com, Hawaii, United States	2002	21 Jan 2014	1	3	4	1
76	Crohns Disease Information: Is Colon Cleansing the Answer, Colon Cleanse Information	http://www.colon-cleanse-information.com/crohns-disease-information.html	Colon-Cleanse-Information.Com, MKR Concepts, Oregon, United States	2007	11 Nov 2014	3	0	5	0
77	Crohn's Disease or Regional Enteritis, MD India.	http://www.medindia.net/patients/patientinfo/Crohns-Disease.htm	Medindia4u.com Pvt. Ltd, Chennai, India	2000	21 Nov 2014	5	0	1	0
78	Crohn's Disease Information, Digestive Disorders	http://www.articleinsider.com/health-and-fitness/digestive-disorders/crohns-disease-information	Digestive Disorders, United States	2003	03 Sep 2014	2	1	2	1
79	Inflammatory Bowel Disease, Patient Center, American College of Gastroenterology	http://patients.gi.org/topics/inflammatory-bowel-disease/	Patient Center, American College of Gastroenterology, Maryland, United States	1996	16 May 2013	4	3	1	2
80	Crohn's Disease: Symptoms, Diagnosis and Treatment, Disabled World. Com	http://www.disabled-world.com/health/digestive/crohns-disease/	Disabled World. Com, New York, United States	2004	29 Oct 2012	1	3	4	1
81	Crohn's Disease, Nutritionist Resource	http://www.nutritionist-resource.org.uk/articles/crohns-disease.html	Nutritionist Resource, Surrey, United Kingdom	2010	9 Feb 15	3	1	3	0

82	Crohn's Disease: Symptoms, Diagnosis and Treatment, verywell.com	http://seniorhealth.about.com/cs/digestivetract/a/crohns_2.htm	Verywell.com part of about.com, Inc., United States	1999	10 Feb 14	6	0	4	0
83	Ulcerative Colitis, Halyard Surgical.	http://www.ulcerative-colitis.org/	Halyard Surgical, New South Wales, Australia	2003	7 Nov 14	2	0	6	1
84	Ulcerative Colitis Overview, Health Communities.com	http://www.healthcommunities.com/colitis/ulcerative-colitis-overview.shtml	Healthcommunities.com, New York, United States	1998	10 May 13	3	2	0	0

IBD: Inflammatory bowel disease; NHS: National Health Service; UNC: University of North Carolina.

Table 2 Summarizes websites included in the study on inflammatory bowel disease included in the study: The accuracy scores (calculated using the DISCERN score and the HONcode score) and the readability scores

No.	Website title, Organisation	URL	Accuracy scores		Readability scores	
			The DISCERN Score (mean ± SD)	The HONcode score (Out of 100)*	The Flesch-Kincaid Grade Level Index	The Coleman-Liau Readability Index
1	Inflammatory Bowel Disease (IBD), Mayo Clinic	http://www.mayoclinic.org/diseases-conditions/inflammatory-bowel-disease/basics/definition/con-20034908	65.0 ± 1.0	0.86*	13.1 ± 0.0	15.0 ± 0.0
2	Inflammatory Bowel Disease Health Center, WebMd	http://www.webmd.com/ibd-crohns-disease/	41.3 ± 1.1	0.70*	10.5 ± 3.2	13.3 ± 2.5
3	Inflammatory Bowel Disease (IBD), Center for Disease Control and Prevention (CDC)	http://www.cdc.gov/ibd/	28.7 ± 0.6	0.90	10.9 ± 2.7	13.3 ± 0.6
4	Inflammatory Bowel Disease, NHS Choices	http://www.nhs.uk/conditions/inflammatory-bowel-disease/pages/introduction.aspx	53.7 ± 0.6	0.63	12.2 ± 1.6	12.3 ± 1.5
5	What are Crohn's and Colitis? Crohn's and Colitis Foundation	http://www.ccfa.org/what-are-crohns-and-colitis/	50.3 ± 0.6	0.81	16.2 ± 9.3	13.7 ± 0.6
6	Inflammatory Bowel Disease, KidsHealth	http://kidshealth.org/parent/medical/digestive/ibd.html	50.7 ± 0.6	0.45	12.0 ± 0.7	11.7 ± 0.6
7	Inflammatory Bowel Disease (IBD). FamilyDoctor	http://familydoctor.org/familydoctor/en/diseases-conditions/inflammatory-bowel-disease.html	45.0 ± 1.0	0.63*	9.6 ± 1.4	10.7 ± 0.6
8	Inflammatory Bowel Disease, Healthline	http://www.healthline.com/health/inflammatory-bowel-disease#Overview1	41.0 ± 1.0	0.59*	8.7 ± 0.6	11.7 ± 0.6
9	Crohn's and Colitis, Australia	https://www.crohnsandcolitis.com.au/about-crohns-colitis/inflammatory-bowel-disease/	41.7 ± 0.6	0.34	13.6 ± 3.2	13.0 ± 1.0
10	Crohn's and Colitis UK	http://www.crohnsandcolitis.org.uk/information-and-support/information-about-ibd/what-is-IBD	60.7 ± 0.6	0.45	10.4 ± 1.6	10.3 ± 3.1
11	Inflammatory Bowel Disease (IBD) (Intestinal Problems of IBD), MedicineNet	http://www.medicinenet.com/inflammatory_bowel_disease_intestinal_problems/article.htm	57.3 ± 1.5	0.75*	13.3 ± 1.5	14.3 ± 1.5
12	Inflammatory Bowel Disease Center, Cedars-Sinai	http://www.cedars-sinai.edu/Patients/Programs-and-Services/Inflammatory-Bowel-Disease-Center/	25.7 ± 1.1	0.27	13.1 ± 2.9	14.0 ± 3.6
13	Inflammatory Bowel Diseases Symptoms and Treatment: Livescience	http://www.livescience.com/39880-inflammatory-bowel-disease.html	39.3 ± 0.6	0.27	11.1 ± 1.4	11.3 ± 1.5
14	Inflammatory Bowel Diseases Program, Penn Medicine.org	http://www.pennmedicine.org/gastroenterology/patient-care/gi-diseases/inflammatory-bowel-disease-ibd/	38.0 ± 0.0	0.54	15.1 ± 2.6	13.0 ± 2.0

15	Inflammatory Bowel Diseases Support Groups, IBDsupport.org	http://www.ibdsupport.org/	52.0 ± 1.7	0.61*	12.8 ± 3.7	13.0 ± 3.6
16	Inflammatory Bowel Disease (IBD), ABC Health and wellbeing	http://www.abc.net.au/health/library/stories/2012/02/22/3435688.htm	34.0 ± 0.0	0.45	11.1 ± 0.3	11.3 ± 0.6
17	Inflammatory Bowel Disease (IBD), GIKids	http://www.gikids.org/content/7/en/IBD	31.0 ± 0.0	0.52	12.5 ± 0.3	12.7 ± 0.6
18	Inflammatory Bowel Disease, Vitamin D Council	https://www.vitamindcouncil.org/health-conditions/inflammatory-bowel-disease/	31.3 ± 1.1	0.43	11.0 ± 10.4	10.7 ± 1.5
19	Inflammatory Bowel Disease Symptoms and Diagnosis, Seattle children's	http://www.seattlechildrens.org/medical-conditions/digestive-gastrointestinal-conditions/ibd-symptoms/	37.3 ± 0.6	0.43	8.9 ± 1.0	11.0 ± 1.0
20	Crohn's Disease, Patient.co.uk	http://www.patient.co.uk/health/crohns-disease-leaflet	55.7 ± 1.5	0.59*	8.5 ± 2.0	10.0 ± 1.7
21	Patient Information Crohn's Disease (Beyond and the Basics), Uptodate	http://www.uptodate.com/contents/crohn-disease-beyond-the-basics	36.7 ± 1.1	0.70	11.2 ± 1.9	12.3 ± 0.6
22	Crohn's Disease, Centre for digestive diseases	http://www.cdd.com.au/pages/disease_info/crohns_disease.html	34.7 ± 0.6	0.52	12.0 ± 0.6	14.3 ± 1.1
23	Crohn's Disease, Patients: British Society for Gastroenterology	http://www.bsg.org.uk/patients/general/crohn-s-disease.html	49.7 ± 0.6	0.56	11.3 ± 1.7	10.7 ± 1.1
24	Crohn's Disease, Bupa	http://www.bupa.co.uk/health-information/directory/c/crohns-disease	52.0 ± 0.0	0.77*	8.2 ± 0.5	9.0 ± 1.7
25	Crohn's Disease, University of Maryland Medical Center	http://umm.edu/health/medical/reports/articles/crohns-disease	64.7 ± 1.5	0.81	15.3 ± 4.8	15.0 ± 1.0
26	Crohn's Disease, Symptoms, Diagnosis, Treatment, Southern Cross	https://www.southerncross.co.nz/AboutTheGroup/HealthResources/MedicalLibrary/tabid/178/vw/1/ItemID/523/Crohns-disease-symptoms-diagnosis-treatment.aspx	42.7 ± 0.6	0.40	13.8 ± 0.3	13.7 ± 0.6
27	Crohn's Disease, American family physician	http://www.aafp.org/afp/2011/1215/p1379.html	42.3 ± 1.1	0.18	9.0 ± 2.9	12.3 ± 4.2
28	What is Crohn's Disease? What Causes Crohn's Disease? MNT	http://www.medicalnewstoday.com/articles/151620.php	45.7 ± 1.5	0.50*	11.2 ± 2.1	10.7 ± 1.5
29	Crohn's Disease, Netdoctor	http://www.netdoctor.co.uk/diseases/facts/crohnsdisease.htm	43.0 ± 0.0	0.59	10.0 ± 1.6	12.3 ± 1.5
30	Crohn's Disease, UCSF medical center	http://www.ucsfhealth.org/conditions/crohns_disease/	41.0 ± 1.0	0.40	11.4 ± 0.5	11.3 ± 0.6
31	Crohn's Disease Symptoms and Treatment, US.news Wellness	http://health.usnews.com/health-news/health-wellness/articles/2013/08/03/crohns-disease-symptoms-and-treatment	48.3 ± 0.6	0.50	6.7 ± 0.8	12.3 ± 2.5
32	What are the treatments for Crohn's disease? Beth Israel Deaconess Medical Center	http://www.bidmc.org/Centers-and-Departments/Departments/Digestive-Disease-Center/Inflammatory-Bowel-Disease-Program/Crohns-Disease/What-are-the-treatments-for-Crohns-disease.aspx	41.7 ± 1.5	0.59	14.3 ± 2.2	14.3 ± 1.1
33	Diagnosing Crohn's, Crohn's and Me.	http://www.crohnsandme.com/crohns-information/crohns-disease-diagnosis.aspx	43.3 ± 1.1	0.27	13.6 ± 1.1	12.7 ± 2.5
34	Understanding Crohn's Disease, Crohn' and Colitis.	http://www.crohnsandcolitisinfo.com/Crohns/What-is-Crohns-Disease	41.7 ± 0.6	0.45	12.4 ± 2.0	12.0 ± 2.6
35	Learning About Crohn's Disease, National Human Genome Research Institute	http://www.genome.gov/25521854	29.0 ± 0.0	0.40	10.8 ± 0.5	12.3 ± 1.5

36	Crohn's Disease, UPMC Life Changing Medicine	http://www.upmc.com/services/digestive-disorders-center/services/ibd/conditions/pages/crohns-disease.aspx	44.3 ± 0.6	0.27	20.3 ± 9.5	15.0 ± 2.0
37	Crohn's Disease, Cincinnati Children's	http://www.cincinnatichildrens.org/health/c/crohns/	44.7 ± 0.6	0.40	9.3 ± 1.1	10.7 ± 0.6
38	Crohn's Disease, Cleveland clinic	http://my.clevelandclinic.org/health/diseases_conditions/hic_Inflammatory_Bowel_Disease_IBD_QandA/hic_Crohns_Disease	26.3 ± 1.1	0.50*	12.9 ± 1.2	12.3 ± 2.3
39	Treatment of Crohn's Disease, UNC Multidisciplinary Center for IBD Research and Treatment	http://www.med.unc.edu/gi/specialties/ibd/about-ibd/treatment-of-ibd-1/treatment-of-crohns-disease	42.3 ± 0.6	0.45	12.3 ± 0.5	13.3 ± 0.6
40	Crohn's Disease, Emedicine health	http://www.emedicinehealth.com/crohn_disease/article_em.htm	59.3 ± 1.2	0.86*	13.0 ± 1.2	13.0 ± 1.7
41	Understanding Crohn's Disease and Ulcerative Colitis. Australian Gastroenterology Institute.	http://www.nevdgp.org.au/info/gastro/crohns.htm	42.3 ± 1.1	0.27	10.4 ± 0.7	11.3 ± 0.6
42	Crohn's Disease, Health Centers	http://www.drweil.com/drw/u/ART00339/Crohns-Disease.html	37.0 ± 0.0	0.50	11.9 ± 1.1	12.7 ± 1.5
43	Crohn's Disease- An Overview, the Royal Children's Hospital Melbourne	http://www.rch.org.au/kidsinfo/fact_sheets/Crohns_Disease_an_overview/	33.7 ± 1.1	0.50	7.7 ± 2.0	11.3 ± 1.5
44	Fighting Inflammatory Bowel Disease Together, the Irish Society for Colitis and Crohn's Disease	http://www.iscc.ie/page.php?id=18&title=What%20is%20IBD	37.7 ± 0.6	0.40	11.6 ± 1.4	10.7 ± 0.6
45	Crohn's Disease Diagnosis, New health guide	http://www.newhealthguide.org/Crohn%27s-Disease-Diagnosis.html	18.3 ± 0.6	0.65	13.5 ± 0.7	13.7 ± 1.1
46	Crohn's Disease, HealthDay.	http://consumer.healthday.com/encyclopedia/digestive-health-14/digestion-health-news-200/crohn-s-disease-644392.html	61.0 ± 1.0	0.86*	10.9 ± 1.4	12.0 ± 0.0
47	Ulcerative Colitis, Wikipedia	http://en.wikipedia.org/wiki/Ulcerative_colitis	54.0 ± 0.0	0.63	15.2 ± 2.1	14.0 ± 2.6
48	Living with UC, Do You Know Your Treatment Options?	http://www.livingwithuc.ca/	44.3 ± 1.1	0.68	12.2 ± 1.6	11.3 ± 2.3
49	What Is Ulcerative Colitis? Everyday Health	http://www.everydayhealth.com/conditions/ulcerative-colitis	40.0 ± 0.0	0.54*	14.1 ± 0.7	13.0 ± 1.0
50	What Is Ulcerative Colitis? News Medical	http://www.news-medical.net/health/What-is-Ulcerative-Colitis.aspx	46.0 ± 1.0	0.59	11.9 ± 0.2	13.0 ± 1.0
51	Crohn's Disease and Ulcerative Colitis, Better Health Channel	http://www.betterhealth.vic.gov.au/bhcv2/bhcarticles.nsf/pages/Crohn%27s_disease_and_ulcerative_colitis	34.3 ± 1.1	0.31	9.8 ± 2.4	11.3 ± 1.5
52	Ulcerative Colitis, Jackson Siegelbaum Gastroenterology	http://gicare.com/diseases/ulcerative-colitis/	52.3 ± 0.6	0.36	10.6 ± 1.4	11.7 ± 2.1
53	Ulcerative Colitis, Crohn's and Colitis Canada	http://www.crohnsandcolitis.ca/site/c.dtJRL9NUJmL4H/b.9012449/k.C223/Ulcerative_Colitis.htm	19.7 ± 0.6	0.31	9.2 ± 1.8	10.0 ± 1.0
54	Ulcerative Colitis, Healthgrades	http://www.healthgrades.com/conditions/ulcerative-colitis	52.7 ± 1.1	0.68	14.5 ± 3.5	14.0 ± 1.0
55	Information for Those with Ulcerative Colitis, Colitis UK	http://www.ulcerativecolitis.org.uk/	49.0 ± 1.7	0.45	14.5 ± 2.0	11.7 ± 1.1

56	Colitis and Chronic Ulcerative Colitis, Virginia Mason	https://www.virginiamason.org/ColitisandChronicUlcerativeColitis	40.7 ± 0.6	0.31	16.7 ± 3.9	15.0 ± 2.0
57	Ulcerative Colitis, GastroNet	http://www.gastro.net.au/diseases/ulcerativecolitis.html	26.7 ± 0.6	0.60	13.1 ± 1.1	13.3 ± 2.3
58	Ulcerative Colitis, Ulcerative Colitis Net.	http://www.ulcerativecolitis.net/	41.3 ± 0.6	0.30	11.4 ± 3.3	12.0 ± 2.0
59	Inflammatory Bowel Disease, Lab Tests Online	http://labtestsonline.org/understanding/conditions/inflammatory-bowel	38.3 ± 0.6	0.32*	13.0 ± 0.7	12.0 ± 1.0
60	Inflammatory Bowel Disease Fact Sheet, Womenshealth.gov	http://www.womenshealth.gov/publications/our-publications/fact-sheet/inflammatory-bowel-disease.html	55.7 ± 1.5	0.40	8.8 ± 0.7	10.0 ± 1.0
61	Inflammatory Bowel Disease (IBD), Innerbody	http://www.innerbody.com/diseases-conditions/ibd	39.0 ± 1.0	0.25	17.2 ± 5.7	16.7 ± 1.5
62	Inflammatory Bowel Disease (IBD), Rightdiagnosis	http://www.rightdiagnosis.com/i/inflammatory_bowel_disease/intro.htm	38.0 ± 1.0	0.33	15.9 ± 4.1	15.7 ± 2.1
63	Inflammatory Bowel Disease, Lifescript.com	http://www.lifescript.com/health/centers/digestive/related_conditions/inflammatory_bowel_disease.aspx	32.0 ± 1.0	0.50	15.1 ± 2.8	12.7 ± 1.5
64	Inflammatory Bowel Disease (IBD), MUSC Health	http://www.ddc.musc.edu/public/symptomsDiseases/diseases/smallBowel/IBD.html	22.7 ± 1.1	0.27	14.2 ± 2.2	15.7 ± 2.9
65	Inflammatory Bowel Disease (IBD): Ulcerative Colitis, Crohn's Disease, New York-Presbyterian Digestive Diseases	http://nyp.org/services/digestive/ibd.html	32.3 ± 0.6	0.27	13.2 ± 0.5	12.7 ± 0.6
66	Inflammatory Bowel Disease, Human Diseases and Conditions Forum.	http://www.humanillnesses.com/original/Her-Kid/Inflammatory-Bowel-Disease.html	35.3 ± 1.5	0.14	10.7 ± 0.9	13.3 ± 2.3
67	Facts About Crohn's Disease, US. Food and Drug Administration	http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm107358.htm	34.7 ± 2.1	0.36	10.6 ± 2.6	13.7 ± 2.1
68	Crohn's Disease Symptoms and warning Signs, SymptomFind	http://www.symptomfind.com/diseases-conditions/crohns-disease-symptoms-warning-signs/	26.0 ± 1.0	0.26*	10.0 ± 1.9	13.7 ± 2.3
69	Crohn's Disease-At a Glance, SixPartsWater. Org	http://www.sixpartswater.org/knowledge-centre/crohns-disease/glance	39.7 ± 0.6	0.15	18.3 ± 3.4	12.7 ± 0.6
70	Ulcerative Colitis, eMedTV	http://colitis.emedtv.com/ulcerative-colitis/ulcerative-colitis.html	55.3 ± 0.6	0.32*	12.1 ± 0.8	11.0 ± 2.0
71	Crohn's Disease, Department of Surgery, University of California.	http://colorectal.surgery.ucsf.edu/conditions--procedures/ulcerative-colitis.aspx	51.0 ± 1.0	0.73	12.4 ± 0.8	12.3 ± 2.5
72	Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases	http://www.niddk.nih.gov/health-information/health-topics/digestive-diseases/crohns-disease/Pages/facts.aspx	68.3 ± 1.2	0.95	13.6 ± 3.9	13.7 ± 5.5
73	Crohn's Disease, Patient Education Center	http://www.patienteducationcenter.org/articles/crohns-disease/	38.0 ± 1.0	0.30	8.9 ± 0.8	12.3 ± 1.5
74	Crohn's Disease Information, alot health	http://health.alot.com/conditions/crohns-disease-information--163	32.3 ± 0.6	0.22	10.4 ± 1.5	12.0 ± 2.6
75	Crohn's Disease, Diagnose-me.Com	http://www.diagnose-me.com/symptoms-of/crohns-disease.html	32.0 ± 1.0	0.28	12.5 ± 1.6	14.3 ± 1.1

76	Crohns Disease Information: Is Colon Cleansing the Answer, Colon Cleanse Information	http://www.colon-cleanse-information.com/crohns-disease-information.html	35.7 ± 2.5	0.36	13.2 ± 0.5	14.7 ± 1.5
77	Crohn's Disease or Regional Enteritis, MD India.	http://www.medindia.net/patients/patientinfo/Crohns-Disease.htm	46.3 ± 1.1	0.54	11.8 ± 1.3	14.0 ± 2.6
78	Crohn's Disease Information, Digestive Disorders	http://www.articleinsider.com/health-and-fitness/digestive-disorders/crohns-disease-information	33.3 ± 0.6	0.60	10.2 ± 0.6	12.3 ± 0.6
79	Inflammatory Bowel Disease, Patient Center, American College of Gastroenterology	http://patients.gi.org/topics/inflammatory-bowel-disease/	58.3 ± 1.2	0.80	12.7 ± 1.3	13.0 ± 1.0
80	Crohn's Disease: Symptoms, Diagnosis and Treatment, Disabled World.Com	http://www.disabled-world.com/health/digestive/crohns-disease/	26.3 ± 2.3	0.19	8.6 ± 0.6	12.00 ± 1.73
81	Crohn's Disease, Nutritionist Resource	http://www.nutritionist-resource.org.uk/articles/crohns-disease.html	39.3 ± 0.6	0.57	10.2 ± 2.6	11.3 ± 1.1
82	Crohn's Disease: Symptoms, Diagnosis and Treatment, verywell.com	http://seniorhealth.about.com/cs/digestivetract/a/crohns_2.htm	44.3 ± 1.5	0.37	12.6 ± 0.7	11.0 ± 1.0
83	Ulcerative Colitis, Halyard Surgical.	http://www.ulcerative-colitis.org/	51.7 ± 1.5	0.25	12.1 ± 0.6	12.7 ± 2.5
84	Ulcerative Colitis Overview, Health Communities.com	http://www.healthcommunities.com/colitis/ulcerative-colitis-overview.shtml	52.7 ± 1.5	0.23*	12.6 ± 1.2	12.7 ± 1.1

*Websites that received HONCode certificates. IBD: Inflammatory bowel disease; NHS: National Health Service; UNC: University of North Carolina.

also varied from a minimum of 0.14 to a maximum of 0.95 (mean ± SD, 0.16 ± 0.19; median = 0.45, IQR = 0.29). The lowest HONcode score was scored by the website, Crohn's Disease, American family physician, while the maximum score was scored by the website, Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases. Along with the HONcode trust worthy scores, HONcode certificate was indicated for websites that have received such certificates, Table 2.

The top ten websites on IBD as per the DISCREN scores were in the following order: The Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases (scored 68), Inflammatory Bowel Disease, MayoClinic (Scored 65), Crohn's Disease, University of Maryland Medical Center (scored 64), Crohn's Disease, HealthDay (scored 61), Crohn's Disease and Colitis UK (scored 60), Crohn's Disease, eMedicine health (scored 59), Inflammatory Bowel Disease, Patient Center, American College of Gastroenterology (scored 58), Inflammatory Bowel Disease, MedicineNet (scored 57), Inflammatory Bowel Disease, Fact Sheet, Womenshealth.gov (scored 55), Ulcerative Colitis, eMedTV (scored 55). The top ten websites as per the HONcode tool were in the following order: Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases (scored 0.95), Inflammatory Bowel Disease, Center for Disease Control and Prevention (scored 0.90), Inflammatory Bowel Disease, MayoClinic (scored 0.86), Crohn's Disease, eMedicine health (scored 0.86), Crohn's Disease, HealthDay (scored 0.86), What are Crohn's & Colitis? Crohn's & Colitis Foundation (scored 0.81), Crohn's Disease, University of Maryland Medical Center (scored 0.81), Inflammatory Bowel Disease, Patient Center, American College of Gastroenterology (scored 0.80), Crohn's Disease, Bupa (scored 0.77), and Inflammatory Bowel Disease, MedicineNet (scored 0.75). It is interesting to note that the website, Crohn's Disease, the National Institute of Diabetes and Digestive and Kidney Diseases was ranked number one as per the two instruments. Seven websites in total were among the top ten websites as per both the DISCERN and the HONcode scores. Nine out of the ten websites were created in United State.

Grouping the websites under five categories

Table 3 summarizes the grouping of the 84 websites under five categories on the basis of the website creators. Universities and research centers created 25 (%), professional foundations and associations created 15 (%), commercial and pharmaceutical companies created 25 (%), charities and volunteers contributed to 9 (%), non-university educational bodies such as colleges, academies, councils, WebMed contributed to 10 (%). Further analysis revealed that there was no

Table 3 Grouping the websites on inflammatory bowel disease included in the study under five categories

Category	Number	DISCERN score		HONcode score	
		mean ± SD[6]	95%CI for means	mean ± SD[7]	95%CI for means
Universities and Research Centers[1]	25	41.3 ± 11.3	36.6-45.9	0.46 ± 0.20	0.37-0.54
Foundations and Associations[2]	15	44.5 ± 10.5	38.6-50.2	0.53 ± 0.16	0.44-0.62
Commercial and Pharmaceutical Companies[3]	25	40.4 ± 10.1	36.3-44.6	0.44 ± 0.18	0.36-0.52
Charities and Volunteer work[4]	9	40.2 ± 11.9	31.0-49.4	0.39 ± 0.17	0.26-0.52
Non-university Educational Bodies[5]	10	46.7 ± 9.7	39.7-53-6	0.63 ± 0.20	0.48-0.76
Total	84	42.1 ± 10.7	39.8-44.5	0.48 ± 0.19	0.44-0.52

[1]This category includes university-affiliated centers, state hospitals, national or state research centers; [2]This category includes gastroenterological societies, foundations, and associations- most were on inflammatory bowel disease, Crohn's disease or ulcerative colitis; [3]This category includes industrial bodies, commercial and pharmaceutical companies aiming at serving the community and patients with inflammatory bowel disease; [4]This category includes charities and websites created by individuals, or groups; [5]This category includes all other non-university educational bodies including colleges, academies, councils, WebMed, *etc.*; [6]The DISCERN scores were not significantly different between the groups as per ANOVA (combined, $P = 0.472$) or (linear term, $P = 0.475$); [7]The HONcode scores were significantly different between the groups as per ANOVA (combined, $P = 0.041$). The linear term, $P = 0.228$.

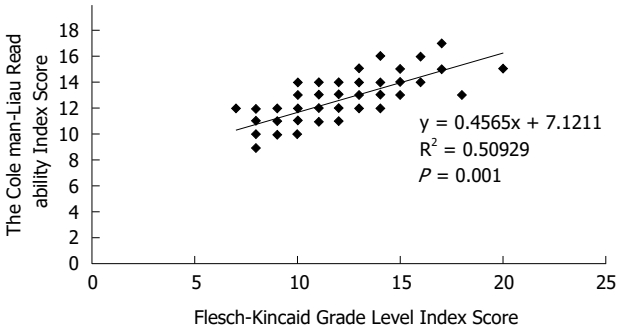

Figure 2 Correlation between the scores of the two readability methods: Coleman-Liau Readability Index and Flesch-Kincaid Grade Level Index.

significant differences in the DISCERN scores between the groups ($P = 0.472$) but the HONcode scores were different ($P = 0.041$). Examples of content deficiencies or scientific content inaccuracies and suggestions for improvement are shown on Table 4.

Readability level of websites

Table 2 summarizes the readability scores calculated by using two methods, the Flesch-Kincaid Grade Level Index and Coleman-Liau Readability Index. The minimum score for the Flesch-Kincaid Grade Level Index was 6.7 for the website Crohn's Disease Symptoms and Treatment, United States news Wellness, while the maximum score was 20.3 for the website Crohn's Disease, UPMC Life Changing Medicine. Out of the 84 websites, 28 received a mean of 6.7 to 10.9, forty-six received a mean of 11.0 to 14.5, and ten websites received a mean of 15.7 to 20.3. The overall mean score for the 84 websites was 11.9 ± 2.4.

For the Coleman-Liau Readability Index the minimum score was 9.0 for the website Crohn's Disease, Bupa, while the maximum score was 16 for the website Inflammatory Bowel Disease, Fact Sheet, Womenshealth. Out of the 84 websites, eleven received a mean score of 9.0 to 10.9, thirty-nine received a score of 11.0 to 12.7, and thirty-four received a score of 13.0 to 16.0. The overall mean score for the 84 websites was 12.6 ± 1.5.

Significant correlation was found between the Flesch-Kincaid Grade Level index scores and the Coleman-Liau Index scores ($R^2 = 0.509$, $P < 0.001$) (Figure 2).

The agreement between the evaluators

Table 5 summarizes the inter-rater agreement between evaluators for the DISCERN instrument items. The overall Cohen kappa scores were in the range of 0.804-0.876.

DISCUSSION

Several studies pointed to continuous progress from paper to electronic and online-based patient education[29,30]. The aims of the study were to evaluate the quality and accuracy of information available on IBD websites and calculate the readability level using two methods. To maximize the yield of the search, we searched three search engines commonly used by the public seeking information related to healthcare. The study showed that the 84 websites identified were created by universities, affiliated hospitals and research centers, professional foundations and associations, commercial and pharmaceutical companies created, charities and volunteers, as well as non-university educational bodies (such as colleges, academies, councils, and WebMed). The involvement of universities, affiliated hospitals, and research centers is directed at health information exchange as well as public and patient education with the aim to improve the quality of care, engage the patient in the decision-making processes and the journey of treatment as well as enhance patient's awareness about the nature of their illness. Such educational approaches while having multiple impacts on the patients' healthcare; it can also help in reducing the costs of treatment[31]. The current move from paper-based to online health care education may be related to the progressive increases in the use of the Internet by the public and patients[32]. Furthermore, Morgan *et al*[33] showed that patients with genetic and chronic diseases have great interest

Table 4 Examples of assessment of the content of some websites on inflammatory bowel disease

Website Number	Title	Areas of deficiencies	Suggestions for improvement
45	Crohn's Disease Diagnosis, New health guide	Symptoms of Crohn's disease are briefly mentioned. Some details are needed to explain the common presenting symptoms. No mention of differential diagnosis. No mention of investigations needed to confirm the diagnosis. Nothing is mentioned about treatment of Crohn's disease.	Symptoms may include abdominal pain, typically in the right lower quadrant, diarrhoea, some blood may be present in stools, fatigue. In more severe disease fever, and weight loss may be present. Some patients may have nausea, and abdominal distention together with abdominal pain. It is worth to mention that Crohn's disease is a lifelong illness (chronic disease). People who have Crohn's will experience periods of flare-ups, when their symptoms are active, and other times when their symptoms go into remission. Up to 30% of patients may have changes in the area around the anus including anal fistulas (internal tracts connecting the anal lumen with the skin around the anus), abscess, skin tags, and anal fissures. About 10%-20% of patients also have joint pains, lower back pain, skin rash known as erythema nodosum, and eye changes. (images showing some of these changes will enhance this part).
53	Ulcerative Colitis, Crohn's and Colitis Canada	Symptoms are briefly stated. No mention of differential diagnosis, investigations and no discussion of medical and surgical treatment.	A section discussing investigations should be added. In addition to detailed medical history, the treating doctor will initiate the evaluation by testing for infectious conditions that can cause inflammation of the colon, screen for endocrine-metabolic disorders such as excessive activity of the thyroid gland. Therefore biochemical tests and stool tests are needed. Endoscopic evaluation (colonoscopy) should be carried out in patients who have symptoms suggestive of inflammatory bowel disease and no evidence for an infection to explain symptoms. Small bowel images, computed tomography (CT) enterography may also be needed. Nutritional changes, medical and surgical treatment should be briefly discussed.
64	Inflammatory Bowel Disease (IBD), MUSC Health	Symptoms of inflammatory bowel disease are not clearly written. One would wonder, are "bowel sores" and "intestinal bleeding" symptoms? Differential diagnosis is not mentioned. The approach for diagnosing inflammatory bowel disease is not mentioned and the treatment of IBD is not explained.	As discussed earlier.
68	Crohn's Disease Symptoms and Warning Signs, SymptomsFind	Although symptoms of Crohn's disease are mentioned briefly, they are not explained. Mild, moderate and severe inflammatory bowel disease are stated but not explained. This should be explained in a simple language. Complications are mentioned but there was no mention how the disease is diagnosed, and what investigations are needed. Nothing is mentioned about nutritional changes, medical and surgical treatment of inflammatory bowel disease.	Patients are described to have mild ulcerative colitis when they have: -Fewer than four bowel motions (stools) per day. -No bleeding or small amounts of bleeding in their stools. -Normal erythrocyte sedimentation rate (ESR) -No fever, no anaemia and no increases in their heart rate, Patients are described to have moderate ulcerative colitis when they have: -More than four stools per day. -Mild elevation in ESR. Patients are described to have severe ulcerative colitis when they have: -More than six stools a day (loose stools). -Fever, rapid heartbeat, and anaemia. -Elevated ESR. The website may also mention changes that necessitate hospital admission and medical attention. Websites may provide key questions that patients may use when they review their treating doctors. Examples of these questions: •I wonder what's causing these symptoms? •What type of tests do I need? Do these tests require any special preparation? •What treatments are available, and which do you recommend? •Are there any medications that I should avoid? •Do I need to follow any dietary restrictions? •Are there any risks if I become pregnant? The MayoClinic website has listed a number of useful questions that patients can use.

12	Inflammatory Bowel Disease Center, cedars-Sinai.	Under symptoms of Crohn's disease, it is written, "The most common signs are pain in the stomach area (usually on the right side) and diarrhea", it is not clear what is meant by pain in the stomach area on the right side? Complications are provided but no signs are stated. No mention of differential diagnosis, no mention of investigations and possible findings. Nutritional changes, medical and surgical treatment are not explained.	The authors should differentiate between symptoms and signs. Scientific errors are noted in the website and common presenting symptoms should be stated. It may be useful to explain the symptoms under two main headings: symptoms in children, and symptoms in adults. Investigations needed to diagnose the disease should be discussed. Patients are usually interested to know more detail about these investigations. Information provided should answer questions such as -Name of the test -Why the test is need? -What is the test about? -Nature of the investigation (invasive *vs* non-invasive) -Are there special preparations needed prior to the test? -Any possible complications related to the investigation? -What can the results of the test tell the patient and the treating doctor? It is also important to state that IBD is an ongoing condition (chronic disease), so some of the tests may need to be repeated from time to time, or extra tests may be needed. These investigations may include: (1) Blood tests including full blood count, inflammatory markers tests including erythrocyte sedimentation rate (ESR), c-reactive protein (CRP), liver function tests, urea and electrolytes, and other biochemical tests, (2) stool tests including stool microscopy, stool culture and sensitivity, fecal markers such as fecal calprotectin, fecal lactoferrin, (3) Endoscopy including colonoscopy, sigmoidoscopy, proctoscopy, with biopsies for histological studies (4) radiological studies such as barium studies, CT scans, MRI scans and PET scans.

Table 5 Summarizes the inter-rater agreement between evaluators calculated using Cohen kappa coefficient scores

DISCERN items	Mean score (95%CI of the difference)			Reviewer variability (κ range)
	Evaluator 1	Evaluator 2	Evaluator3	
1. Are the aims clear?	1.7 (1.5-2.0)	1.7 (1.5-2.0)	1.8 (1.5-2.0)	0.885-0.953
2. Does it achieve its aims?	1.4 (0.9-1.8)	1.5 (1.0-1.9)	1.5 (1.0-1.9)	0.790-0.904
3. Is it relevant?	3.8 (3.6-4.0)	3.8 (3.6-4.0)	3.8 (3.6-4.0)	0.792-0.887
4. Is it clear what sources of information were used to compile the publication (other than the author or producer)?	2.5 (2.2-2.9)	2.5 (2.2-2.8)	2.5 (2.2-2.8)	0.879-0.880
5. Is it clear when the information used or reported in the publication was produced?	2.7 (2.4-3.1)	2.7 (2.3-3.0)	2.7 (2.3-3.0)	0.793-0.875
6. Is it balanced and unbiased?	3.7 (3.5-4.0)	3.8 (3.6-4.0)	3.7 (3.5-3.9)	0.796-0.890
7. Does it provide details of additional sources of support and information?	2.2 (1.9-2.5)	2.2 (1.9-2.5)	2.2 (1.9-2.5)	0.792-0.827
8. Does it refer to areas of uncertainty?	3.2 (3.0-3.4)	3.1 (3.0-3.3)	3.2 (3.0-3.3)	0.764-0.832
9. Does it describe how each treatment works?	3.1 (2.8-3.4)	3.1 (2.8-3.4)	3.1 (2.8-3.4)	0.859-0.874
10. Does it describe the benefits of each treatment?	2.4 (2.2-2.7)	2.4 (2.2-2.7)	2.5 (2.2-2.8)	0.782-0.841
11. Does it describe the risks of each treatment?	2.3 (2.0-2.6)	2.3 (2.0-2.6)	2.3 (2.0-2.7)	0.772-0.902
12. Does it describe what would happen if no treatment is used?	1.5 (1.3-1.7)	1.5 (1.3-1.7)	1.5 (1.3-1.8)	0.870-0.923
13. Does it describe how the treatment choices affect overall quality of life?	2.0 (1.8-2.2)	2.0 (1.8-2.2)	2.1 (1.9-2.3)	0.859-0.906
14. Is it clear that there may be more than one possible treatment choice?	3.6 (3.4-3.9)	3.6 (3.4-3.9)	3.6 (3.4-3.8)	0.773-0.849
15. Does it provide support for shared decision-making?	2.7 (2.4-2.9)	2.8 (2.6-3.0)	2.8 (2.5-3.0)	0.767-0.854
16. Based on the answers to all of the above questions, rate the overall quality of the publication as a source of information about treatment choices?	2.9 (2.7-3.2)	3.0 (2.7-3.2)	2.9 (2.6-3.2)	0.900-0.959

in participating in clinical studies and a desire to understand information discussed during reviewing their healthcare provider. These patients may have more questions after they leave the doctor's clinic and usually tend to search the Internet for answers[33]. Compared to paper-based health education, the Internet appears to provide a wider range of answers and options. However, the quality of information provided and the readability level remain as areas of concern[8,34].

As per this study, the DISCERN and the HONcode scores varied. However, no significant differences in the DISCERN scores were found between the groups but when the groups were compared on the basis of the HONcode scores, the difference was significant. A weak correlation was found between the DISCERN scores and the HONcode scores (R^2 = 0.217). The results are consistent with the variability of the DISCERN scores of websites in each group and the fact that the two instruments are not measuring the same characteristics[25]. Interestingly, seven out of the top 10 websites on IBD scored higher on both the DISCERN and the HONcode scales. Looking into the readability levels of these seven websites, the readability using the Flesch-Kincaid Grade level was in the range 11 to 15, while for the Coleman-Liau Readability Index the range was 12 to 15. This indicates that even the top 7 websites had a readability level not adjusted to the public level.

Out of the 84 websites, only 17 displayed the HONcode certificate. A recent study found that only three websites out of 78 showed HONcode certificates[35]. Although the number of websites granted a HONcode certificate is small yet there is no correlation between the calculated HONcode scores and having a certificate on the website. Absence of the HONcode certificate from a website doesn't necessarily indicate poor quality of the website. This is because the process of issuing the HONcode is based on a voluntary application for the certificate. Therefore, it is possible that the owners/authority responsible for these websites did not apply for the HONcode certificate.

The readability scores were calculated by using two methods, the Flesch-Kincaid Grade Level Index and Coleman-Liau Readability Index. The moderate correlation between the Flesch-Kincaid Grade Level index scores and the Coleman-Liau Index scores is consistent with other work[20] and indicates that the results from the two calculations are consistent. The findings show that the majority of the studies had a readability level equivalent to year 11 and 12. However, the national reading grade level average has been estimated to be about the 6th-grade[36] and the general agreement is that the reading level for patient information materials should not exceed this level and be no less than what a 4th-grade is capable of reading[37]. With these findings in mind, there is a need for editing the content of most websites identified and adjusting the reading levels to meet the recommended reading levels for the public.

This study has a number of strengths; first, we searched three different search engines commonly used by the public seeking health-related information with the aim to maximize the yield of the search. Second, we used two instruments the DISCERN and the HONcode to measure the accuracy of contents. Both instruments have been widely used in assessing online health information material. Third, three evaluators independently conducted the evaluation and the inter-rater agreement among the assessors was within the accepted limits. Finally, the readability was measured by using two different methods. However, this study is not without limitations; the study is just a representation of websites identified at the time of the search. Only websites in the English language were included, and there is the possibility that there are other websites in other languages that match with our inclusion criteria and were not included. A multinational study may be needed to identify any differences if any and resolve gaps in this area. Therefore, despite all efforts and the plans considered, we may have missed some websites.

The results of this study may be of value to general practitioners, physicians, gastroenterologists, nurses, and allied health professionals, the public and medical students interested in online education material on IBD. The top 10 websites with the highest DISCERN and the HONcode scores identified from this study provide examples of educationally useful websites that can be recommended by treating physicians to their patients. However, their readability level was above the recommended level for the public and they may be suitable for educated patients only.

Future directions

This study highlights a number of future directions in research in the area of Internet-based patient education particularly patients with IBD. These can be summarized as follows: First, planning for creating online educational material for the public and patients with IBD necessitates more care for innovation, content accuracy and readability level to match the recommended needs of the public. Second, more work is needed to enhance the use of images, illustrations, and videos in improving the educational usefulness of websites on IBD and engage the patients and the public using such online resources. The use of these educational tools should aim at explaining difficult concepts, and enhancing understanding of the message given. As shown from this study the use of these educational tools was deficient in most websites. Third, future research should aim at assessing the impact of using Internet education and health literacy in patients with IBD and whether such resources have made impacts on number of hospital admissions, costs associated with poor health literacy, effective health education techniques, and how poor health literacy influences management outcome in these patients.

In conclusion, health literacy about IBD and the use of Internet as a medium for education appears to be increasing. Universities, research centers, commercial and pharmaceutical companies, professional foundations and associations were the major contributors to online resources written for the public and patients. Several deficiencies in the content were observed and most websites failed to meet the recommendations set by the National Institute of Health and American Medical Association that patients resources should be written about the 6th-grade level. Effective use of

diagrams, illustrations, videos, and tables to explain difficult concepts should be encouraged. Revising the websites and resolving the gap between the readability of written health information and the literacy skills of the public will improve the purpose of these websites and make them a useful healthcare resource to patients with IBD.

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COMMENTS

Background

Patients with inflammatory bowel disease (IBD), as it is the case with other chronic diseases, seek information about the nature of their disease. The increasing use of the Internet embraces the significance of online resources educating patients and the public.

Research frontiers

With the abundance of information there is concern about the quality, accuracy and readability level of information available on the web, thus it might be useful to assess the quality of these resources, identify specific deficiencies and examine whether these websites meet the recommendations of national bodies.

Innovations and breakthrough

The goal of this paper is to use comprehensive analysis to assess the quality of websites, accuracy of content and readability levels of websites on IBD dedicated to patients and the public.

Applications

The study highlights a number of future directions in the area of Internet-based patient education particularly patients with IBD and raises the need for improving such resources particularly in relation to specific areas identified in the study.

Terminology

Scientific accuracy and quality of content were evaluated using two standardised instruments widely used in research. The readability level was calculated on the bases of word length, sentence length and syllables.

Peer-review

This paper is an interesting evaluation of quality, accuracy, and readability of websites dedicated to the public. It is a novelty and represents a beginning point for judging and improving websites dedicated to IBD.

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SYSTEMATIC REVIEWS

Diabetes mellitus, insulin resistance and hepatitis C virus infection: A contemporary review

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Abstract

AIM

To summarise the literature data on hepatitis C virus (HCV)-infected patients concerning the prevalence of glucose abnormalities and associated risk.

METHODS

We conducted a PubMed search and selected all studies found with the key words "HCV" or "hepatitis C virus" and "diabetes" or "insulin resistance". We included only comparative studies written in English or in French, published from January 2000 to April 2015. We collected the literature data on HCV-infected patients concerning the prevalence of glucose abnormalities [diabetes mellitus (DM) and insulin resistance (IR)] and associated risk [*i.e.*, severe liver fibrosis, response to antivirals, and the occurrence of hepatocellular carcinoma (HCC)].

RESULTS

HCV infection is significantly associated with DM/IR compared with healthy volunteers and patients with hepatitis B virus infection. Glucose abnormalities were associated with advanced liver fibrosis, lack of sustained virologic response to interferon alfa-based treatment and with a higher risk of HCC development. As new antiviral therapies may offer a cure for HCV infection, such data should be taken into account, from a therapeutic and preventive point of view, for liver and non-liver consequences of HCV disease. The efficacy of antidiabetic treatment in improving the response to

antiviral treatment and in decreasing the risk of HCC has been reported by some studies but not by others. Thus, the effects of glucose abnormalities correction in reducing liver events need further studies.

CONCLUSION

Glucose abnormalities are strongly associated with HCV infection and show a negative impact on the main liver related outcomes.

Key words: Hepatitis C virus; Diabetes mellitus; Insulin resistance; Liver fibrosis; Treatment

Core tip: Hepatitis C virus (HCV) infection is associated with increased rates of glucose abnormalities, including diabetes mellitus and insulin resistance. The presence of glucose abnormalities in HCV infected patients, including diabetes mellitus and insulin resistance, is associated with negative liver-related outcomes (*i.e.*, severe liver fibrosis, decreased response to antivirals, and increased occurrence of hepatocellular carcinoma).

Desbois AC, Cacoub P. Diabetes mellitus, insulin resistance and hepatitis C virus infection: A contemporary review. *World J Gastroenterol* 2017; 23(9): 1697-1711 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1697.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1697

INTRODUCTION

Hepatitis C virus (HCV) infection is a major health problem. The World Health Organization (WHO) estimates that at least 150-170 million people, approximately 3% of the world's population, are chronically infected. These patients are known to be at risk of liver related complications, *i.e.*, cirrhosis and hepatocellular carcinoma (HCC), with an estimated liver-related mortality of 350000 people/year. The total risks of morbidity and mortality are underestimated, because they do not take into account extrahepatic consequences of HCV infection. Numerous extrahepatic manifestations have been reported, suggesting that HCV is more a systemic disease than just a liver disorder. In large prospective cohort studies, up to two-thirds of patients with HCV infection experienced extra-hepatic manifestations[1]. The majority of available data concern HCV-related autoimmune and/or lymphoproliferative disorders, from benign mixed cryoglobulinemia to frank lymphomas, which is consistent with HCV lymphotropism[2]. More recently, other HCV-associated disorders have been reported including cardiovascular, renal, central nervous system and metabolic diseases[3]. Among the latter, some studies assessed the risk of diabetes mellitus (DM) or insulin resistance (IR) while others evaluated the impact of DM/IR on the main liver-related HCV infection outcomes (*i.e.,* liver fibrosis, cirrhosis, HCC). However, the results appear to be conflicting, with great heterogeneity between studies.

In the present study, based on a literature data review, we aimed to analyse: (1) the risk of glucose abnormalities (GA) in HCV-infected patients; and (2) the impact of GA on the main liver-related HCV outcomes, *i.e.,* liver fibrosis, response to interferon alfa-based treatment, and HCC.

MATERIALS AND METHODS

We conducted a PubMed search and selected all studies found with the key words "HCV" or "hepatitis C virus" and "diabetes" or "insulin resistance". We included only comparative studies written in English or in French, published from January 2000 to April 2015. We selected surveys that had evaluated the risk of Type 2 DM or IR in HCV-infected patients compared with healthy controls or with patients with hepatitis B virus (HBV) infection. The definition of DM was usually based on a fasting plasma glucose > 1.26 g/L, or a history of diabetes mellitus, or use of oral antidiabetic agents or insulin. The definition of IR was based on the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) according to the formula: HOMA-IR = fasting glucose (mmol/L) × fasting insulin (mIU/L)/22.5. We also included studies that assessed the association between the presence of glucose abnormalities (DM or IR) and the main HCV infection outcomes (*i.e.,* liver fibrosis, cirrhosis, response to antiviral treatment, HCC). Conversely, studies that evaluated the impact of antiviral treatment on glucose abnormalities were included. We excluded studies with patients infected with the HBV or human immunodeficiency virus, and those for whom the entire manuscript was not available.

RESULTS

Is HCV infection associated with an increased prevalence of glucose abnormalities?

We included two types of studies: (1) those that assessed the HCV prevalence in diabetic patients compared with non-diabetics; and (2) studies that assessed the prevalence of DM and/or IR in HCV-infected patients compared with controls (healthy volunteers or HBV carriers) (Table 1).

Six studies evaluated HCV prevalence rates in diabetic patients compared with non-diabetic healthy volunteers. The number of participants ranged from 180 to 13000. Four out of the six studies showed a significant increased prevalence of HCV infection markers [HCV antibodies (n = 3), HCV RNA (n = 1)] in DM patients, with an odds ratio (OR) between 2.87 and 3.03[4-7]. Of note, only one study used multivariate

Table 1 Glucose abnormalities and hepatitis C virus infection

Ref.	Year	Country	Study design	Patients	Patients number	Controls	Controls number	Testing for HCV Ab or RNA	Endpoint	Statistical methods	Association	Statistics
HCV infection markers in patients with type 2 diabetes mellitus												
Sangiorgio *et al*[4]	2000	Italy	Retrospective	DM	1514	HV	1300	Ab	HCV	Univariate	Yes	$P < 0.0001$
Chen *et al*[5]	2006	Taiwan	Cross sectional	DM	820	HV	905	Ab	HCV	Univariate adjusted	Yes	OR = 2.87 [1.51, 5.46]; $P < 0.001$
Huang *et al*[6]	2007	Taiwan	Cross sectional	DM	1237	HV	8595	RNA	HCV	Univariate	Yes	6.9% *vs* 4.5%; $P < 0.001$
Jadoon *et al*[7]	2010	Pakistan	ND	DM	3000	HV	10000	Ab	HCV	Univariate	Yes	OR = 3.03 [2.64, 3.48]; $P = 0.001$
Balogun *et al*[53]	2006	Nigeria	case-control	DM	90	HV[2]	90	Ab	HCV	Univariate	No	NS
Costa *et al*[54]	2008	Brazil	Case-control	DM	206	HV	206	RNA	HCV	Multivariate	No	NS
Glucose abnormalities in HCV infected patients *vs* different control groups												
Vs healthy volunteers												
Knobler *et al*[17]	2000	Israel	Case-control	HCV	45	HV[2]	88	RNA	DM	Univariate	Yes	33% *vs* 5.6%; $P < 0.001$
Mehta *et al*[8]	2000	United States	Cross sectional	HCV	230	HV	9611	Ab	DM	Multivariate	Yes	OR = 3.77 [1.8, 7.87]
Marzouk *et al*[18]	2007	Egypt	Cross sectional	HCV	190	HV	575	RNA	DM	Multivariate	Yes	HR = 3.05 [1.19, 7.81]
Shaheen *et al*[19]	2007	United States	ND	HCV	239	HV	10144	ND	IR	Univariate adjusted	Yes	OR = 1.68; $P = 0.02$
Huang *et al*[6]	2007	Taiwan	Cross sectional	HCV	478	HV[2]	7927	RNA	DM	Multivariate	Yes	OR = 1.53 [1.18, 1.98]; $P < 0.001$
Huang *et al*[21]	2008	Taiwan	ND	HCV	683	HV[2]	515	RNA	DM/IGT[1]	Univariate	Yes	OR = 3.51 [2.7, 4.56]; $P < 0.001$
Park *et al*[20]	2008	South Korea	Prospective	HCV[1]	62	HV[2]	172	RNA	IR	Univariate	Yes	22.5% *vs* 5.2%; $P < 0.001$
Mohamed *et al*[22]	2009	Egypt	Cross sectional	HCV[1]	38	HV[2]	12	RNA	IR	Univariate	Yes	HOMA-IR = 3.98 (normal ALT) and 2.69 (a normal ALT) *vs* 1.92; $P < 0.001$
Duseja *et al*[23]	2009	India	ND	HCV[1]	85	HV[2]	25	RNA	IR	Univariate	Yes	62% *vs* 16%; $P = 0.0002$
Lonardo *et al*[24]	2009	Italy	ND	HCV[1]	97	HV	182	RNA	IR	Univariate	Yes	$P < 0.001$
Huang *et al*[25]	2009	Taiwan	ND	HCV[1]	93	HV	144	Ab	IR	Univariate	Yes	HOMA-IR 2.2 *vs* 1.6; $P = 0.02$
Mostafa *et al*[26]	2010	Egypt	ND	HCV	329	HV	173/795	RNA	DM	Univariate adjusted	Yes	OR = 1.35 [1.06, 1.73]; $P = 0.02$
Miyajima *et al*[27]	2013	Japan	Cross sectional	HCV	40	HV	1780/88	RNA	IR	Univariate	Yes	HOMA-IR 3.0 *vs* 1.3; $P < 0.001$
Younossi *et al*[28]	2013	United States	Retrospective	HCV	177	HV	19568	RNA	DM and IR	Multivariate	Yes	OR for DM 2.3 [1.18, 4.54] OR for IR 2.06 [1.19, 3.57]
Pothineni *et al*[29]	2014	United States	Retrospective	HCV	1434	HV[2]	14799	RNA	DM	Univariate	Yes	11.2% *vs* 5.1%; $P < 0.01$
Dai *et al*[30]	2013	Taiwan	Retrospective	HCV	160	HV[2]	480	RNA	DM	Multivariate	Yes	OR = 1.208 [1.009, 2.799]; $P = 0.004$
Mehta *et al*[10]	2003	United States	Case-control	HCV	12	HV[2]	1072	RNA	DM	Univariate	No	NS
Stepanova *et al*[11]	2012	United States	Nationwide survey	HCV	791	HV	38715	RNA	DM and IR	Multivariate	No	NS
Montenegro *et al*[9]	2013	Italy	Prospective	HCV	616	HV	1856	Ab	DM	Univariate adjusted	No	NS
Ruhl *et al*[55]	2014	United States	Cross sectional	HCV	277	HV	14571	RNA	DM	Univariate adjusted	No	NS
Vs hepatitis B virus infection												
Knobler *et al*[17]	2000	Israel	Case-control	HCV	45	HBV	90	RNA	DM	Univariate	Yes	33% *vs* 12%; $P = 0.004$
Ryu *et al*[31]	2001	South Korea	Prospective	HCV, F4	68	HBV	157	Ab	DM	Univariate	Yes	24% *vs* 10.4%; $P = 0.001$
Wang *et al*[32]	2007	Taiwan	Longitudinal	HCV	926	HBV	544	Ab	DM	Multivariate	Yes	HR = 1.7
Huang *et al*[6]	2007	Taiwan	Cross sectional	HCV	478	HBV	1363	RNA	DM	Univariate	Yes	18% *vs* 11.4%; $P < 0.001$
Moucari *et al*[33]	2008	France	Retrospective	HCV	500	HBV[2]	100	RNA	HOMA-IR	Univariate	Yes	35% *vs* 5%; $P < 0.001$
White *et al*[12]	2008	United States	Meta-analysis	HCV	34 studies	HBV/HV	-	Ab/RNA	DM	Meta-analysis	Yes	Adjusted OR for HV 1.68 and for HBV 1.80
Rouabhia *et al*[34]	2010	Algeria	Prospective cross sectional	HCV[1]	290	HBV	126	RNA	DM	Multivariate	Yes	OR = 4.73 [1.7, 13.2]; $P = 0.0029$

Petta *et al*[56]	2011	Italy	Retrospective	HCV	170	HBV[2]	170	RNA	HOMA-IR and DM	Univariate	Yes	42.2% *vs* 25.9%, $P = 0.002$ and 8.8% *vs* 3.6%, $P = 0.04$
Imazeki *et al*[57]	2008	Japan	Retrospective	HCV	544	HBV	286	RNA	DM and IR	Multivariate	No	NS
Tanaka *et al*[58]	2008	Japan	Case-control	HCV[1]	30	HBV[2]	30	RNA	IR	Multivariate	No	NS
Mavrogiannaki *et al*[59]	2008	Greece	prospective case control	HCV	108	HBV	81	RNA	glucose intolerance	Univariate adjusted	No	NS
Persico *et al*[60]	2009	Italy	Retrospective	HCV	726	HBV	126	Ab	DM	Univariate adjusted	No	NS

[1]HCV infection not treated; [2]Matched for confounding factors (age and/or gender and/or BMI and/or ALT…). HCV: Hepatitis C virus infection; Ab: Antibody; HV: Healthy volunteers; G1: Genotype 1; SVR: Sustained virological response; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; IR: Insulin resistance; DM: Diabetes mellitus; FPG: Fasting Plasma glucose; IGT: Impaired glucose tolerance [after oral glucose tolerance test (OGTT)]; CLD: Chronic liver disease; NAFLD: Non-alcoholic fatty liver disease; NS: Not significant; ND: Not determined.

logic regression analysis, while another adjusted the risk for age, gender, body mass index (BMI) and alanine aminotransferase (ALT) levels. One study showed an increased HCV antibody prevalence rate in DM patients with abnormal ALT levels.

Thirty-two studies evaluated DM and/or IR prevalence rates in HCV patients compared with either healthy volunteers ($n = 20$) or HBV patients ($n = 12$). The size of cohorts ranged from 50 to 39506 subjects. All but four studies assessed DM/IR prevalence in HCV-RNA positive patients. In 10 out of 20 studies that compared HCV patients with healthy volunteers, multivariate or univariate analyses with adjustment for age, gender, BMI, socio-economic status and ethnicity were performed. Thirteen studies evaluated DM prevalence ($n = 11$) or occurrence ($n = 2$), while others ($n = 9$) assessed IR in HCV infected patients. Overall, 16 out of 20 studies found a significant association between the presence of glucose abnormalities (DM/IR) and HCV infection, including 7 out of 10 studies with multivariate or adjusted analyses (OR between 1.2 and 3.77). One study reported a higher risk of DM only in patients older than 40 years[8]. Four studies reported "negative" results. Three out these four studies showed a higher risk of DM only in specific populations (*i.e.*, HCV patients with increased ALT levels[9], HCV patients older than 55 years with a BMI > 25 kg/m^2[10], and a cohort studied between 1988 and 1994, but not in the more recent cohort)[11].

When compared with HBV infected patients, 7 out of 11 studies found a significant association of HCV with DM. In one meta-analysis[12], a positive HCV viremia was associated with an increased risk of DM compared with controls (adjusted OR = 1.68) and with HBV patients (adjusted OR = 1.80).

Are diabetes mellitus or insulin resistance associated with liver fibrosis severity in HCV infected patients?

Thirty studies investigated whether DM/IR was associated with liver fibrosis severity in HCV patients (Table 2). Studies were performed in Asia (Taiwan $n = 3$, Japan $n = 3$, other $n = 1$), Europe ($n = 13$), the United States and Australia ($n = 5$), Saudi Arabia ($n = 1$), Turkey ($n = 1$) and Egypt ($n = 3$). The mean size of the cohorts was 451 patients (min-max range 10 to 3068). The authors searched for an association between liver fibrosis severity and DM ($n = 9$), IR ($n = 19$) or impaired fasting plasma glucose ($n = 2$). All but two studies performed multivariate analyses. Twenty-six out of thirty studies reported a significant association of glucose abnormalities with liver fibrosis severity (OR from 1.28 to 13.72). Three of the four "negative" studies were done on small cohorts. There were some differences related to HCV genotypes, but no systematic relationship was found.

Do diabetes mellitus and insulin resistance have an impact on the virological response to HCV treatment?

Twenty-six studies and three meta-analyses investigated whether GA had an impact on the response to interferon alfa-based antiviral treatment (Table 3). The studies originated from Europe ($n = 11$), Asia ($n = 4$), Egypt ($n = 4$), the United States ($n = 5$), Australia ($n = 1$) and Saudi Arabia ($n = 1$). They included a mean of 503 patients (50 to 5944). Nineteen out of twenty-eight studies showed a significant negative effect of GA in response to interferon alfa-based therapy [*i.e.,* lower sustained viral response (SVR) rates], including 15 multivariate analyses and 3 meta-analyses. Of note, studies that did not find an impact of GA on SVR rates had some limitations, including small size of cohorts (60-600 patients), only G1 or G4 patients (3 out of 10 studies), and only Italian patients (4 out of 10). Two of them evaluated patients treated with peginterferon/ribavirin and telaprevir. The three meta-analyses found a significant association between IR and the absence of SVR, regardless of the genotype (OR for G1 = 2.2, G2 = 3, G3 = 4.45 and G4 = 6.7, respectively).

Table 2 Glucose abnormalities and severe liver fibrosis in hepatitis C virus-infected patients

Ref.	Year	Country	Number of HCV patients	Patient profile	Glucose abnormality	Statistical method	Association with severe fibrosis[1]	Genotypes	Statistics
Konrad *et al*[42]	2000	Germany	10	Non DM	FPG	Multivariate	Yes	All	$P = 0.01$
Sud *et al*[61]	2004	Australia	170	-	HOMA-IR	Multivariate	Yes	All	OR = 1.47 [1.14, 1.89]; $P = 0.003$
Muzzi *et al*[62]	2005	Switzerland	221	Non DM	HOMA-IR	Multivariate	Yes	All (except G3)	OR = 1.57 [1.04, 2.39]
D'souza *et al*[63]	2005	United Kingdom	59	-	HOMA-IR	Multivariate	Yes	All	$P = 0.001$
Taura *et al*[64]	2006	Japan	83	-	HOMA-IR	Multivariate	Yes	All	OR = 7.32 [1.59, 33.73]; $P = 0.01$
Leandro *et al*[65]	2006	Italy	3068	-	DM	Multivariate	Yes	G1	OR = 4.52 [1.07, 19.1]; $P = 0.011$
Bugianesi *et al*[66]	2006	Italy	132	G3 with steatosis	HOMA-IR	Multivariate	Yes	G3	OR = 2.98 [1.13, 7.89]; $P = 0.028$
Kita *et al*[67]	2007	Japan	68	Post tranfusion hepatitis	DM	Multivariate	Yes	All	OR = 8.4 [2.23, 31.54]; $P = 0.002$
Petta *et al*[68]	2008	Italy	201	G1	DM	Multivariate	Yes	G1	OR = 2.69 [1.46, 4.95]; $P < 0.001$
Moucari *et al*[33]	2008	France	500	-	HOMA-IR	Multivariate	Yes	All	OR = 1.8 [1.16, 2.81]; $P = 0.009$
Cua *et al*[69]	2008	Australia	346	G1, G3, untreated	IR	Multivariate	Yes	G3	OR = 3.15 [1.56, 6.35]; $P = 0.001$
Hsu *et al*[70]	2009	Taiwan	528	G1, G2	FPG	Multivariate	Yes	G1	OR = 13.72 [2.15, 87.7]; $P < 0.05$
Moucari *et al*[71]	2009	France	226	G4	HOMA-IR	Multivariate	Yes	G4	OR = 3.86 [1.859, 8.034]; $P < 0.001$
Persico *et al*[60]	2009	Italy	726	-	DM	Multivariate	Yes	All	$P < 0.05$
Hung *et al*[14]	2011	Taiwan	1470	-	DM	Univariate	Yes	All	$P < 0.001$
Patel *et al*[72]	2011	Asia	263	G2, G3	HOMA-IR	Multivariate	Yes	G2 and G3	OR = 8.42 [2.1, 34.3]; $P = 0.003$
Mohamed *et al*[73]	2011	Egypt	50	G4	HOMA-IR	Multivariate	Yes	G4	OR = 3.73; $P = 0.001$
Miyaaki *et al*[74]	2011	Japan	171	-	DM	Multivariate	Yes	All	OR = 8.739 [2.85, 26.85]; $P = 0.0002$
Conjeevaram *et al*[75]	2011	United States	341	G1	HOMA-IR	Multivariate	Yes	G1	OR = 1.28 [1.07, 1.51]; $P = 0.005$
Petta *et al*[56]	2011	Italy	170	G1	HOMA-IR	Multivariate	Yes	G1	OR = 2.64 [1.11, 6.28]; $P = 0.02$
Khattab *et al*[76]	2012	Egypt	107	G4	HOMA-IR	Multivariate	Yes	G4	OR = 1.87 [1.09, 8.29]; $P = 0.04$
Ziada *et al*[77]	2012	Egypt	140	Non DM	HOMA-IR	Multivariate	Yes	All	OR = 1.92 [0.97, 3.4]; $P = 0.049$
Thompson *et al*[13]	2012	United States	1038	Non DM	HOMA-IR	Multivariate	Yes	All	OR = 1.6 [1.1, 2.33]; $P = 0.02$
Alfaleh *et al*[78]	2013	Saudi Arabia	157	-	DM	Multivariate	Yes	All (except G4)	OR = 0.37 [0.148, 0.927]; $P = 0.034$
Dokmeci *et al*[79]	2014	Turkey	104	-	HOMA-IR	Multivariate	Yes	All	OR = 3.36 [1.32, 31.25]; $P = 0.021$
Huang *et al*[80]	2015	Taiwan	1077	-	DM	Multivariate	Yes	All	OR = 1.81 [1.14, 2.65]; $P = 0.002$
Fartoux *et al*[81]	2005	France	141	Non DM	HOMA-IR	Univariate	No	No	NS
Elgouhari *et al*[82]	2008	United States	183	-	DM	Multivariate	No	No	NS
Petta *et al*[83]	2009	Italy	156	Non DM	HOMA-IR	Multivariate	No	No	NS
Rueger *et al*[84]	2014	Switzerland	1461	-	DM	Multivariate	No	No	NS

[1]Severe liver fibrosis: F3 or F4 in Metavir scoring system. HCV: Hepatitis C virus infection; G1: Genotype 1; SVR: Sustained virological response; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; IR: Insulin resistance; DM: Diabetes mellitus; FPG: Fasting plasma glucose; NS: Not significant.

What is the impact of interferon alfa-based treatment on glucose abnormalities?

Twenty studies assessed the impact of interferon-based antiviral treatment on DM/IR, either as an improvement of GA after treatment or as the occurrence of GA after antiviral treatment (Table 4).

Improvement of GA after antiviral treatment was analysed in fifteen surveys that included 13 to 1038 HCV treated patients. Most of these studies performed univariate analyses. A significant decreased prevalence of GA was noted in 12 out of 15 studies. Eleven of these 12 studies reported a significant change of IR

Table 3 Impact of glucose abnormalities on virological response after interferon alpha based treatment

Ref.	Year	Country	Patients number	Patient profile	Association	Statistical method	Impact on virological response	Genotypes	Statistics
D'souza *et al*[63]	2005	United Kingdom	59		HOMA-IR	Multivariate	Yes	All	OR of SVR: 0.44 [0.22, 0.88]; $P = 0.02$
Tarantino *et al*[85]	2005	Italy	80		GMI	Univariate	Yes	All	40% *vs* 7.5%; $P = 0.0009$
Romero-Gomez *et al*[86]	2005	Spain	159		HOMA-IR	Multivariate	Yes	All	OR of SVR 0.55 [0.33, 0.93]; $P = 0.012$
Jian Wu *et al*[87]	2006	China	98		HOMA-IR	Multivariate	Yes	All	OR of SVR: 0.17; $P = 0.015$
Backus *et al*[88]	2007	United States	5944	G1, G2, G3	DM	Multivariates	Yes	All and G1	OR = 0.76 [0.64, 0.71]; $P = 0.002$
Conjeevaram *et al*[89]	2007	United States	401	G1	HOMA-IR	Multivariates	Yes	G1	OR = 0.87 [0.77, 0.99]; $P = 0.028$
Elgouhari *et al*[82]	2008	United States	183		DM	Multivariate	Yes	All	OR of SVR 0.22 [0.07, 0.55]; $P = 0.003$
Poustchi *et al*[90]	2008	Australia	82	G2, G3 non DM	HOMA-IR	Multivariate	Yes	G2, G3	OR of SVR 0.16 [0.03, 0.77]; $P = 0.02$
Romero-Gomez *et al*[91]	2008	Spain	1059		FPG	Multivariate	Yes	All	OR of SVR 0.56 [0.34, 0.93]; $P < 0.02$
Moucari *et al*[71]	2009	France	226	G4	HOMA-IR	Multivariate	Yes	-	OR of SVR: 0.19 [0.07, 0.51]; $P = 0.001$
Dai *et al*[92]	2009	Taiwan	330	G1, G2	HOMA-IR	Multivariate	Yes	G1, G2	OR of SVR 0.872 [0.79, 097]; $P = 0.01$
Hung *et al*[115]	2010	Taiwan	1470		DM	Multivariate	Yes	All	OR of SVR 0.69 [0.5, 0.96]; $P = 0.029$
Khattab *et al*[93]	2010	Egypt	131	Non DM, G4	HOMA-IR	Multivariate	Yes	G4	OR of SVR 0.07 [0.01, 0.43]; $P = 0.004$
Deltenre *et al*[94]	2011	France	2732	G1-6	IR	Meta-analysis	Yes	All	-
Eslam *et al*[95]	2011		2129	G1-6	IR	Meta-analysis	Yes	All	OR of SVR 0.35 [0.24, 0.51]; $P = 0.0004$
Del Campo *et al*[96]	2012	Spain	240	Non DM	HOMA-IR	Multivariate	Yes	G1, G4	OR of SVR 0.44 [0.17, 0.97]; $P = 0.04$
Ziada *et al*[77]	2012	Egypt	140	Non DM	HOMA-IR	Multivariate	Yes	All	OR of SVR 0.41 [0.18, 0.9]; $P = 0.003$
Laurito *et al*[97]	2013	Brazil	2238	G1-6	IR	Meta-analysis	Yes	All	OR of SVR 0.41 [0.3, 0.56]; $P = 0.022$
Abd El-Wahab *et al*[98]	2014	Egypt	392	Non DM	HOMA-IR	Multivariate	Yes	All	OR of virological response: 0.19 [0.1, 0.38]; $P = 0.0001$
Grasso *et al*[99]	2009	Italy	90	Non DM, G1	HOMA-IR	Multivariate	No	G1	NS
Fattovich *et al*[100]	2010	Italy	412		HOMA-IR	Multivariate	No	No	NS
Khattab *et al*[76]	2012	Egypt	107	G4	HOMA-IR	Multivariate	No	G4	NS
Brandman *et al*[101]	2012	United States	23	Non DM	IGT, FGP, SSGP	Univariate	No	No	NS
Aghemo *et al*[102]	2012	Italy	339		HOMA-IR	Univariate	No	No	NS
Fattovich *et al*[100]	2012	Italy	124	Non DM	HOMA-IR	Multivariate	No	No	NS
Serfaty *et al*[103]	2012	France	161[1]	G4	HOMA-IR	Multivariate	No	G4	NS
Alfaleh *et al*[78]	2013	Saudi Arabia	157		DM	Multivariate	No	No	NS
Younossi *et al*[104]	2013	United States	578[1]	G1	HOMA-IR	Univariate adjusted	No	G1	NS
Jung *et al*[105]	2014	Soutk Korea	60		HOMA-IR	Univariate	No	No	NS

[1]Treated with peginterferon/ribavirin telaprevir. HCV: Hepatitis virus infection; G1: Genotype 1; SVR: Sustained virological response; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; IR: Insulin resistance; IGT: Impaired glucose tolerance; DM: Diabetes mellitus; FPG: Fasting plasma glucose; SSGP: Steady-state plasma glucose; GMI: Glucose metabolism impairment; NS: Not significant; ND: Not determined.

only in patients who achieved a SVR. One survey found a significant change of IR after antiviral treatment only in genotype 1 patients[13].

Five studies evaluated the risk of GA occurrence according to antiviral treatment response. They included 202 to 2842 HCV treated patients, and all performed multivariate analyses. Four out of five studies showed a significant association between GA occurrence and the absence of SVR.

Do glucose abnormalities increase the risk of HCC in HCV infected patients?

Sixteen studies assessed the association between HCC and DM/IR in HCV infected patients (Table 5). These studies included from 120 to 5186 HCV patients, both treated and non-treated. Most of them (10/16)

Table 4 Glucose abnormalities after interferon alpha based treatment

Ref.	Year	Country	Number of HCV patients	Patient profile	Glucose metabolism parameter	Statistical method	Significant association or difference	Genotypes	Statistics
Improvement of glucose abnormalities after HCV treatment									
Konrad *et al*[42]	2000	United States	13		FPG and FI	Univariate	Yes	All	$P < 0.05$ and $P < 0.01$
Romero-Gomez *et al*[86]	2005	Spain	50		HOMA-IR	Univariate	Yes	All	In SVR; $P < 0.05$
Kawaguchi *et al*[106]	2007	Japan	89		HOMA-IR	Univariate	Yes	All	In SVR; $P < 0.01$
Chehadeh *et al*[107]	2009	Kuwait	181	G4	FPG	Univariate	Yes	G4	In SVR; $P < 0.001$
Kim *et al*[108]	2009	Korea	28	G1, G2	HOMA-IR	Multivariate	Yes	G1, G2	In SVR, OR of decreased IR 50 [3.74, 668.35]; $P = 0.003$
Conjeevaram *et al*[75]	2011	United States	341	G1	HOMA-IR	Univariate	Yes	G1	In SVR; $P < 0.001$
Khattab *et al*[76]	2012	Egypt	107	G4, non cirrhotic	HOMA-IR	Univariate	Yes	G4	In SVR ; $P = 0.001$
Thompson *et al*[13]	2012	United States	1038		HOMA-IR	Multivariate[1]	Yes	All	In G1 SVR; $P = 0.007$
Serfaty *et al*[103]	2012	France	161	G1, non cirrhotic	HOMA-IR	Univariate	Yes	G1	In SVR ; $P < 0.05$
Ziada *et al*[77]	2012	Egypt	140	Non DM, non cirrhotic	HOMA-IR	Univariate	Yes	All	$P = 0.009$
Chan *et al*[109]	2013	Australia	86	Non DM	HOMA-IR	Univariate	Yes	All	In SVR; $P = 0.04$
Jung *et al*[105]	2014	South Korea	60		HOMA-IR	Univariate	Yes	All	In SVR; $P = 0.036$
Mello *et al*[110]	2006	Brazil	30	G1, G3	HOMA-IR	Univariate	No	All	NS
Kawaguchi *et al*[111]	2009	Japan	72	Non DM, non cirrhotic	HOMA-IR, SI and ISI	Univariate[1]	No	No	HOMA-IR: NS In SVR, SI $P = 0.002$ and ISI $P = 0.009$
Brandman *et al*[101]	2012	United States	23	Non cirrhotic	SSGP	Univariate	No	No	NS
Occurrence of glucose abnormalities after HCV treatment									
Simó *et al*[112]	2006	Spain	234	Non DM	DM or IGT	Multivariate[1]	Yes	All	In SVR, OR = 0.48 [0.24, 0.48]; $P = 0.04$
Romero-Gomez *et al*[91]	2008	Spain	1059		DM or IGT	Multivariate[1]	Yes	All	In SVR, OR = 0.44 [0.2, 0.97]; $P = 0.04$
Arase *et al*[113]	2009	Japan	2842		DM	Multivariate[1]	Yes	All	In SVR, HR = 0.36 [0.24; 0.56]
Aghemo *et al*[102]	2012	Italy	339	Non DM	HOMA-IR	Multivariate[1]	Yes	All	In SVR, OR = 0.36 [0.18, 0.72]; $P = 0.004$
Giordanino *et al*[114]	2008	Italy	202	Non DM	DM or IGT	Multivariate[1]	No	No	NS

[1]Association with SVR. HCV: Hepatitis C virus infection; G1: Genotype 1; SVR: Sustained virological response; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; IR: Insulin resistance; DM: Diabetes mellitus; FPG: Fasting plasma glucose; FI: Fasting insulin; IGT: Impaired glucose tolerance; ISI: Insulin sensitivity index; SI: Serum insulin; SSGP: Steady-state plasma glucose; NS: Not significant.

included Asian patients, and all but one performed multivariate analyses.

Five studies looked for the presence of DM/IR in HCV infected patients with HCC compared with HCV patients without HCC. Four out of five studies found a significant association between DM/IR and HCC (as compared with non-HCC) (OR from 2.0 to 11.6).

Nine out of eleven other studies found a significant association between the presence of DM/IR and the development of HCC in the follow-up of HCV infected patients (HR from 1.10 to 6.9). One study found a higher risk of HCC in diabetic patients only with SVR and without cirrhosis[14], while 2 others reported an increased risk of HCC only in diabetic patients with advanced fibrosis[15,16].

DISCUSSION

Many studies have evaluated the association between HCV chronic infection, insulin-resistance and diabetes mellitus. The abnormalities of carbohydrate metabolism, including hyperinsulinemia and IR, known to be *per se* related to chronic hepatic diseases, were the rationale for speculation on this relationship. Insulin-resistance is an often undetected condition, commonly coexisting with obesity and metabolic syndrome, and possibly progressing to type 2 diabetes. HCV-related type 2 diabetes mellitus may arise from a complex interaction between IR, steatosis and inflammatory processes. Epidemiologic studies supporting the association between type 2 diabetes and HCV infection were

Table 5 Glucose abnormalities and hepatocellular carcinoma in hepatitis C virus-infected patients

Ref.	Year	Country	Patient number	Patient profile	Association	Statistical method	Association DM and HCC	Statistics
Diabetes mellitus/insulin resistance in HCV-related HCC								
K-Kutala *et al*[15]	2014	France	162	HCC, not treated for HCV	DM and HCC	Multivariate	Yes[3]	HR = 3.13 [1.17, 8.38]; *P* = 0.022[3]
Hung *et al*[115]	2010	Taiwan	188	59 HCC; 129 non-HCC	DM and HCC	Multivariate	Yes	OR = 11.6 [2.500, 53.800]; *P* = 0.002
Hung *et al*[115]	2010	Taiwan	188	59 HCC; 129 non-HCC	HOMA-IR and HCC	Multivariate	Yes	OR = 2.0 [1.35, 3]; *P* = 0.001
Khattab *et al*[116]	2012	Egypt	294	147 HCC; 147 non-HCC	HOMA-IR and HCC	Multivariate	Yes	OR = 2.5 [1.7, 3.69]; *P* = 0.001
Mohamed *et al*[73]	2011	Egypt	100	50 HCC; 50 non-HCC; 20 non HCV	HOMA-IR and HCC	Univariate	No	NS
Diabetes mellitus/insulin resistance and development of HCC in HCV-infected patients								
Chen *et al*[117]	2008	Taiwan	1095	-	DM and HCC	Multivariate	Yes	OR = 3.52 [1.29, 9.24]
Veldt *et al*[16]	2008	Europe	541		DM and HCC	Multivariate	Yes[3]	OR = 3.28 [1.35, 7.97]; *P* = 0.009[3]
Konishi *et al*[118]	2009	Japan	197	Non DM, treated for HCV	DM[1] and HCC	Multivariate	Yes	HR = 4.63 [1.677, 12.766]; *P* = 0.003
Hung *et al*[14]	2010	Taiwan	1470	Treated for HCV	DM and HCC	Multivariate	Yes[2]	HR = 4.32 [1.23, 15.25]; *P* = 0.023[2]
Nkontchou *et al*[119]	2010	France	248	Cirrhotics	HOMA-IR and HCC	Multivariate	Yes	HR = 1.10 [1.01, 1.21]; *P* = 0.026
Takahashi *et al*[120]	2011	Japan	203	Non DM, treated for HCV	DM[1] and HCC	Multivariate	Yes	HR = 6.9 [1.7, 28.4]; *P* < 0.05
Arase *et al*[121]	2013	Japan	4302	Non treated for HCV	DM and HCC	Multivariate	Yes	HR = 1.73 [1.3, 2.3]; *P* < 0.001
Elkrief *et al*[45]	2014	France	348	Cirrhotics	DM	Multivariate	Yes	HR = 1.938 [1.129 , 3.328]; *P* = 0.016
Toyoda *et al*[122]	2015	Japan	522	Patients with SVR	DM and HCC	Multivariate	Yes	HR = 2.08 [1.0170, 4.0133]; *P* = 0.045
Lai *et al*[123]	2006	Taiwan	2141	-	DM and HCC	Multivariate	No	NS
Chen *et al*[124]	2013	Taiwan	5186	-	DM and HCC	Multivariate	No	NS

[1]Association of abnormal post-challenge hyperglycaemia and HCC; [2]Only in SVR patients without cirrhosis; [3]Only in advanced liver fibrosis. HCV: Hepatitis virus infection; HCC: Hepatocellular carcinoma; SVR: Sustained virological response; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; IR: Insulin resistance; DM: Diabetes mellitus; NS: Not significant.

first published in the early 1990s. More recently, larger epidemiologic studies gave more in-depth analyses of the relationship between HCV chronic infection and glucose abnormalities and were included in the present analysis.

HCV infection is associated with increased rates of glucose abnormalities, including diabetes mellitus and insulin resistance

In the present analysis, most studies found a significant association between HCV infection (whether active HCV RNA positive, or not *i.e.,* HCV Ab positive) and diabetes mellitus or insulin resistance. This tight association was confirmed in both directions by the increased rates of HCV infection markers in DM/IR patients and the high rates of glucose abnormalities in HCV infected patients. The consistency of this association was supported by the confirmation of such results compared with different control groups, such as healthy volunteers or HBV carriers[6,8,12,17-34]. The variability of HOMA-IR cut-offs used (between 1.8 and 2.5 generally) may explain the heterogeneous results reported in the literature. Confounding factors might have also led to significant bias. Indeed, some studies comparing HCV patients with healthy volunteers did not perform multivariate analysis or adjust for confounding factors. However, seven out of ten multivariate analyses found a significant increased risk of DM/IR in HCV patients (OR = 1.2-3.7), after adjusting for confounding variables such as age, gender, BMI, ethnicity and education level.

How are we able to explain the increased risk of DM in HCV infected patients? Some authors have suggested that diabetic patients might have been infected by HCV due to injections or nosocomial transmission. The association of HCV infection with IR and the widespread use of universal precautions nowadays in hospitals to avoid virus transmission probably dis-

qualify this hypothesis. There are a variety of other possible mechanisms of increased risk of DM/IR in HCV patients. As shown in this study, glucose abnormalities in HCV patients are associated with liver fibrosis severity. Severe liver fibrosis and cirrhosis are well-known conditions that are able to induce glucose metabolism impairment. However, studies with other liver diseases, including cirrhosis, still showed an excess of risk in HCV patients compared with HBV patients[6,12,17,31-34]. The ability of HCV, particularly genotype 3 viruses, to induce liver steatosis on its own, which might in turn increase the risk of DM/IR, has also been suggested in previous studies[35,36]. Other underlying mechanisms may involve HCV *per se*. Experimental data suggest the role of inflammation. Increased HOMA-IR has been correlated with soluble Tumor Necrosis Factor Receptor1 (sTNFR1) and sTNFR2 levels[37]. Increased abnormal HOMA-IR was not associated with elevated serum levels of TNFα, IL6 and adiponectin in another study[38]. Other studies have also suggested an impairment of glucose uptake in HCV-infected patients. Glucose uptake and the surface expression of Glucose Transporters (GLUT1 and 2) were suppressed in cells infected by HCV compared with controls[39]. Interferon alfa restored glucose uptake, GLUT2 surface expression, mRNA expression and GLUT2 promoter activities. HCV has also been shown to impair glucose uptake and to promote IR by increasing suppressor of cytokine signalling 3 (SOC3), which inhibits insulin phosphorylation of AKT and phosphoinositide 3-kinase (PI3K)[40]. HCV may be involved in the regulation of phosphorylation of insulin receptor substrate 1 (ISR-1), implicated in the insulin pathway[41]. In HCV core transgenic mice, the viral protein was able to induce increasing TNFα levels in the liver, which in turn promoted the induction of IR. The high levels of TNFα inhibited the ISR-1, causing IR and its possible progression to diabetes. A decreased expression of ISR-1 and ISR-2 mediated by ubiquitination was observed and was inversely proportional to the liver fibrosis stage.

Interferon alfa use might lead to glucose metabolism impairment and is a potential bias. However, increased DM/IR rates have been also reported in HCV patients not taking interferon alfa[20,22-25,34]. Many studies found a decreased rate of glucose abnormalities in HCV patients who showed a SVR after interferon alfa-based therapy, and even in non virological responders in one study[42]. This strongly suggests a direct/indirect role of HCV on glucose metabolism impairment. As eradication of HCV seems to be effective in decreasing the occurrence rate of DM/IR, it will very be interesting to analyse the impact of new direct antiviral agents (DAAs) for preventing DM/IR and eventually cardiovascular disorders. Indeed, in a recent study, IFN-free antiviral regimen resulted in rapid changes in serum lipid profiles and intrahepatic expression of lipid-related genes in G1 patients[43].

Presence of glucose abnormalities in HCV infected patients, including diabetes mellitus and insulin resistance, is associated with negative liver-related outcomes

Severe liver fibrosis, the absence of SVR after interferon alfa-based treatment, and the development of HCC are the main negative outcomes of chronic HCV infection. Interestingly, the presence of DM or IR in HCV patients showed a pejorative impact on each of these end points. Most studies found an independent association of glucose abnormalities with advanced liver fibrosis, absence of SVR after antiviral treatment and HCC occurrence. Only few studies did not confirm such associations. This might be explained by the small size cohort of such studies, the heterogeneity of criteria for DM or HOMA-IR and the very high prevalence of other metabolic risk factors (such as elevated BMI) which may underestimates the impact of DM/IR. Our data is consistent with recent studies that demonstrated that DM increases cumulative incidence of decompensated cirrhosis[44]. In another recent survey, diabetes was independently associated with transplantation-free survival, development of ascites, renal dysfunction, bacterial infections, and HCC during the follow-up[45].

Experimental data suggest that increased insulin levels after hyperglycaemia leads to interferon signalling impairment. Insulin may inhibit the ability of interferon alfa to block HCV replication due to the activation of PI3K by insulin, thus leading to inhibition of STAT-1, which is involved in the interferon alfa pathway[40].

The impact of glucose abnormalities on virological response needs to be further evaluated with new DAA, interferon-free combinations. To date, there is very few data on the impact of GA on virological response to new DAA. Preliminary results suggest that the presence of diabetes does not appear to be predictive of treatment failure in G1 patients[46,47]. Further studies are needed to confirm these data and to evaluate the impact of DM on SVR in patients without poor prognostic factors.

Should glucose abnormalities be corrected to increase SVR rates?

A prospective study, including 155 HCV genotype 1 patients with IR, showed no difference in SVR rates after peginterferon alfa and ribavirin were given, regardless of whether or not patients had received pioglitazone, an antidiabetic drug[48]. Of note, most glycemic control indexes improved significantly in the pioglitazone group except for HbA1c. Another study found higher SVR rates in G4 patients treated with pioglitazone[49]. Pioglitazone may alter NK cell functions and thus impair clearance of infected hepatocytes[48]. A retrospective cohort from Taiwan (19349 diabetic patients, 1.7% HCV positive) showed that patients taking metformin and thiazolidinediones had the lowest risk of HCC (HR 0.49 and 0.56, respectively)

after adjusting for age, gender and comorbidities[50]. Consistently, in a prospective cohort of 100 HCV patients with ongoing cirrhosis, metformin treatment was independently associated with a decrease of HCC occurrence and liver-related death or transplantation[51]. In a two-year prospective follow-up of 85 patients with HCV-related HCC, HCC recurrence-free survival was increased in diabetics taking pioglitazone *vs* non-treated diabetics (44.2% *vs* 36.5%, respectively, $P = 0.37$)[52]. A significant decrease in HCC recurrence was observed in the pioglitazone group for patients with a BMI > 24.

We acknowledge some limitations of this study. Although we tried to include all published studies, we may have missed others in non-English literature or data only presented at meetings. Some studies were done with a limited number of patients. For some studies included in the present analysis, it is possible that there are some remaining bias and residual confounding factors. Despite multivariate analyses, the association between glucose abnormalities improvement and improved outcome may have been influenced by unmeasured confounding factors. Such final confirmation should arise from controlled clinical trials with long-term follow-up.

In conclusion, HCV chronic infection is associated with an increased risk of DM or IR, by a likely direct effect on glucose metabolism. In such patients, DM and IR are associated with a pejorative liver-related prognosis, as shown by increased rates of severe liver fibrosis, HCC occurrence, and decreased SVR rates after interferon-based therapy. This tight relationship between DM/IR and HCV infection needs to be further analysed with new DAAs, interferon-free combinations, with special attention to improvement in glucose abnormalities and long-term follow-up.

COMMENTS

Background

During hepatitis C virus (HCV) infection, extra-hepatic disorders are very frequent and polymorphous. Studies that have evaluated the link between glucose metabolism impairment and HCV reported heterogeneous data.

Research frontiers

Further studies are needed to evaluate the impact of glucose abnormalities in patients treated with interferon-free antiviral therapies. The effects of correction of glucose abnormalities in reducing liver event rates also need to be further studied.

Innovations and breakthroughs

This systematic review allows clarifying the close relationship between glucose abnormalities, HCV infection and poor liver outcomes. HCV infection is associated with increased rates of glucose abnormalities, including diabetes mellitus and insulin resistance. The presence of glucose abnormalities in HCV infected patients, including diabetes mellitus and insulin resistance, is associated with negative liver-related outcomes (*i.e.*, severe liver fibrosis, decreased response to antivirals, and increased occurrence of hepatocellular carcinoma).

Applications

These data strongly encourage clinicians to systematically screen HCV-infected patients for the presence of glucose abnormalities. Considering the impact of glucose abnormalities on liver-related outcomes in HCV infected patients, antiviral treatment should also be considered in HCV-infected patients with metabolic syndrome.

Peer-review

This review talks about the relationship between HCV infection and glucose abnormalities. There are already lots of articles about the topic. This review summarizes those articles published from January 2000 to April 2015 in PubMed and gives us a conclusion about the topic.

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SYSTEMATIC REVIEWS

Presacral venous bleeding during mobilization in rectal cancer

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Abstract

AIM

To analyze the anatomy of sacral venous plexus flow, the causes of injuries and the methods for controlling presacral hemorrhage during surgery for rectal cancer.

METHODS

A review of the databases MEDLINE® and Embase™ was conducted, and relevant scientific articles published between January 1960 and June 2016 were examined. The anatomy of the sacrum and its venous plexus, as well as the factors that influence bleeding, the causes of this complication, and its surgical management were defined.

RESULTS

This is a review of 58 published articles on presacral venous plexus injury during the mobilization of the rectum and on techniques used to treat presacral venous bleeding. Due to the lack of cases published in the literature, there is no consensus on which is the best technique to use if there is presacral bleeding during mobilization in surgery for rectal cancer. This review may provide a tool to help surgeons make decisions regarding how to resolve this serious complication.

CONCLUSION

A series of alternative treatments are described; however, a conventional systematic review in which optimal treatment is identified could not be performed because few cases were analyzed in most publications.

Key words: Presacral hemorrhaging; Rectal surgery; Sacral venous plexus; Pelvic surgery; Sacral anatomy

Core tip: This is a review of 58 published articles on presacral venous plexus injury during the mobilization

of the rectum and on techniques used to treat presacral venous bleeding. We believe that this work is potentially relevant to helping surgeons understand the physiopathology of this complication and making them aware of possible surgical strategies for its treatment.

Casal Núñez JE, Vigorita V, Ruano Poblador A, Gay Fernández AM, Toscano Novella MA, Cáceres Alvarado N, Pérez Dominguez L. Presacral venous bleeding during mobilization in rectal cancer. *World J Gastroenterol* 2017; 23(9): 1712-1719 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1712.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1712

INTRODUCTION

Presacral venous plexus injury during the mobilization of the rectum is one of the most frequent intraoperative complications during rectal cancer surgery[1]. With an incidence that ranges from 0.25% to 8.6%[2,3], it can cause rapid hemodynamic instability in the patient and can even be lethal[4]. The presacral venous plexus cannot be visualized by the surgeon, and injury to the presacral fascia or avulsion of the rectosacral fascia from its insertion into the sacral periosteum can injure the presacral and basivertebral veins, causing bleeding that is difficult to manage with conventional hemostatic maneuvers. Accordingly, a series of techniques that offer alternatives to traditional hemostatic methods for the treatment of presacral venous bleeding have been described. This review aimed to analyze the anatomy of the sacral venous plexus (SVP), the factors influencing the incidence of presacral venous plexus injury, and the flow of bleeding in an effort to classify the available treatment techniques and make surgeons aware of possible strategies for the management of this complication.

MATERIALS AND METHODS

The databases MEDLINE®, PubMed®, and Embase™ were searched for manuscripts published between January 1960 and June 2016 using the following keywords: presacral bleeding, presacral hemorrhage, pelvic surgery, rectal surgery, presacral venous plexus, presacral anatomy, and pelvic packing. The reference lists from the articles were reviewed to identify additional pertinent articles. This review includes 58 articles on the anatomical vascular data of the SVP, the essential factors that influence the flow of bleeding after venous injury, the causes and types of injury, the incidence of presacral bleeding, and treatments applied to control this bleeding in rectal cancer surgery. Due to the limited number of cases reported in most publications and the variety of procedures used to control this complication, conventional systematic review and meta-analysis could not be performed.

RESULTS

Anatomical considerations

The vascular anatomy of the SVP is complex and includes a wide and intricate network of veins primarily formed by the anastomosis between the medial and lateral sacral veins. The medial sacral vein usually drains into the left common iliac vein, whereas the lateral veins drain into the internal iliac vein. The SVP receives contributions from the lumbar veins of the posterior abdominal wall and the basivertebral veins that pass through the sacral foramen. Morphological studies of human sacral bones show that 100% of the specimens feature foramina that communicate with the anterior sacral face and the cancellous bone of the vertebral bodies. Between 16% and 22% of these foramina are 2 to 5 mm in diameter, are located on the anterior face of S4-S5, and are penetrated by the basivertebral veins originating in the cancellous bone, which measures between 0.7 mm and 1.5 mm in this region[4,5] (Figure 1). The small basivertebral veins, which are very thin, allow the bidirectional passage of blood because they lack valves; these veins flow in long, tortuous channels through the spongy tissue of the vertebral bodies. The lateral sacral veins, the medial sacral vein, and the basivertebral veins constitute a wide network of anastomoses that form the venous plexus on the anterior sacral surface[4,6] (Figure 2). The medial sacral vein can be located to the left or the right of the midline and is duplicated in 80% of cases[7]. The vascular anastomoses between the medial sacral vein and the lateral veins are often less than 3 cm from the sacral promontory; specifically, this distance is 2 cm in 90% of cases, and the anastomosis is located at the level of the 3rd and 4th sacral foramen in 70% of cases[6,7]. The retrosacral fascia, also called Waldeyer's fascia, has been described as a sheet of connective tissue that extends from the periosteum of the sacrum to the posterior wall of the rectum approximately 3-4 cm above the anorectal junction. Anatomical and radiological studies have revealed that although its insertion into the sacrum can occur between the 1st coccygeal vertebra and S2, it is located at the level of S3 and S4 in 84%-94% of cases[8-11], just where the foramen that give rise to the basivertebral veins are thickest.

Hydrodynamic studies

The essential factors that influence the flow of blood from an injured vein are the size of the vein and the intravenous pressure at the broken point of the vein. Hydrostatic pressure in the SVP depends on the following: the pressure of the inferior vena cava, the distance from S4-S5 to the coronal axis of the inferior vena cava traced from the renal veins to the iliac bifurcation, and the elevated pressure on the inferior vena cava due to the lithotomy position. Experimental studies and the application of general hydrodynamic principles suggest that the hydrostatic pressure in the

Figure 1 Sacrum specimen. Multiple sacral basivertebral vein foramin, between 2-4 mm, are seen on S4-S5.

sacral plexus in the lithotomy position is approximately twice the venous pressure of the inferior vena cava in the supine decubitus position, and injury to a vein with diameter between 0.5 mm and 4 mm can cause blood flow of 32 mL/min to 1994 mL/min[4,5].

Causes types of injury

Although the height of the tumor in the rectum, the infiltration of the presacral fascia by the tumor, the use of adjuvant radiotherapy, prior rectal surgery, and poor visualization of the surgical field have been described as risk factors that influence the incidence of presacral bleeding during rectal resection, the most common cause is the anatomical relationship of the anorectal fascia. The fascia and its surrounding tissues, including the presacral veins, can be lacerated by the surgeon due to inadequate dissection of the posterior wall of the rectum in the sacral concavity. This maneuver can be caused instrumentally and, more frequently, by blunt dissection by the fingers of the surgeon. The average distance between the ventral surface of the sacrum and the mesorectum is 12 mm or 13 mm as measured by magnetic resonance (MR) and computed tomography (CT), respectively[12]. Laceration of the presacral fascia due to dissection of the sacrum very close to the surface and lifting of this fascia with or without the periosteum are other common causes of presacral bleeding[3,4,13-15].

Wang *et al*[4] describe 3 types of venous injury and direct implications for their handling: injury to the presacral veins (type Ⅰ), injury to the presacral veins and/or basivertebral veins of diameter < 2 mm (type Ⅱ), and injury to the presacral veins and/or basivertebral veins of diameter > 2 mm (type Ⅲ).

Surgical management

In addition to the application of temporary direct pressure on the bleeding area as the first maneuver, various methods have been employed to treat this complication. Ligature of the internal iliac artery is not effective and can cause gluteal and vesical necrosis[16], and ligation of the internal iliac vein makes venous drainage of its tributaries difficult, increases pressure on the sacral plexus, and exacerbates bleeding[4,16-18]. Similarly, Celentano *et al*[19] we propose a classification of techniques (Table 1) and an algorithm for the management of sacral venous bleeding (Figure 3).

Table 1 Classification of techniques for the control of presacral bleeding

Pelvic plugging	
	Traditional with compresses
	Sengstaken-Blakemore tube
	Linton balloon
	Compartmental hemostatic balloon
	IV Saline Bag
	Breast implant
	Plugging with rectus abdominis muscle
	Plugging with Bonewax®
	Plugging with bone cement
	Bakri balloon
Metal implants	
	Simple pins
	Helical titanium pins + Surgicel®
	Staples + cancellous bone + Surgicel®
	Ligaclips®
Topical hemostatic agents	
	Cyanoacrylate
	Cyanoacrylate + Surgicel®
	Ankaferd Blood Stopper®
	Floseal® + Surgicel®
Direct suture	
	Infrarenal aorta clamp + PVS suture
	Suture-circular ligature
Direct/indirect electrocoagulation	
	Spray electrocautery
	Bipolar coagulation
	Argon coagulation
	Electrocoagulation on a piece of epiploic appendix/muscle fragment

Pelvic plugging

Traditional plugging with compresses has been demonstrated to be effective[20], and surgeons should be familiar with this procedure because it may be the only successful mechanism to control potentially fatal bleeding[21]. After abdominoperineal resection, plugging can be performed through the abdomen following closure of the perineum or entirely *via* the perineum. In this case, explantation will require one or more additional laparotomies, and if the perineal route is used, re-bleeding after the explantation will complicate hemostatic maneuvers. The risk of re-bleeding, the increase in infection, the predisposition to dehiscence if the plug is placed adjacent to an anastomosis, and longer hospital admission are the main disadvantages of this procedure[2,14,20,22-24].

Alternatives to classic plugging that attempt to avoid re-intervention have been described, such as the use of an expandable pelvic prosthesis[25] or the use of the Sengstaken-Blakemore probe[26]. Holman

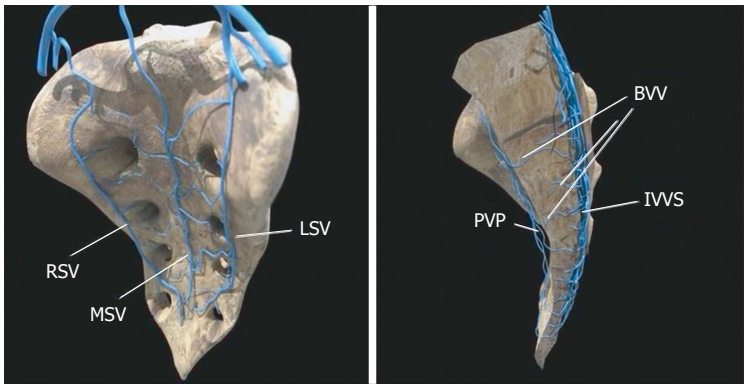

Figure 2 Diagram showing the sacral venous system. RSV: Right sacral vein; LSV: Left sacral vein; MSV: Middle sacral vein; PVP: Presacral venous plexus; IVVS: Internal vertebral venous system; BVV: Basivertebral vein.

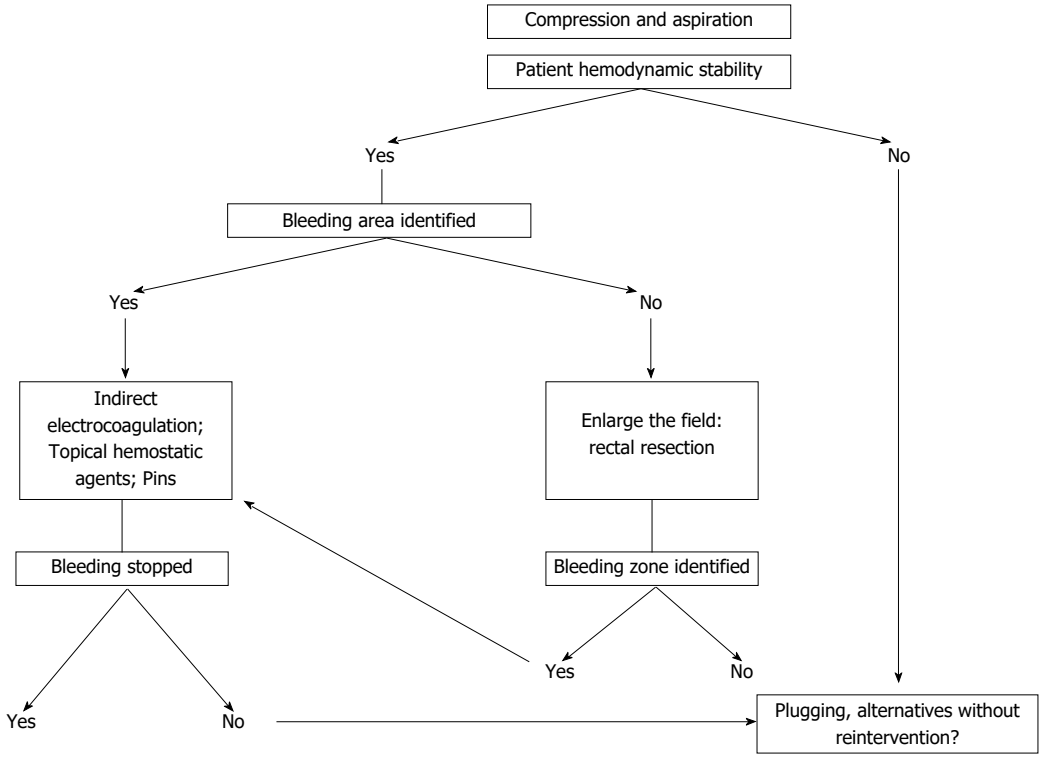

Figure 3 Presacral venous hemorrhaging: treatment algorithm.

et al[27] developed a model hemostatic balloon using MR images of the pelvis. After testing in cadavers, the balloons were used to treat 9 patients with presacral venous bleeding and produced good results in 89% of patients. Ng *et al*[28] describe an alternative to classic plugging after abdominoperineal resection using an empty IV bag filled with 850 mL of saline inserted through the perineum. The advantages of this technique include its adaptability to the sacral concavity and the ease of modifying the hemostatic pressure by infusing or withdrawing fluid through the infusion port. Moreover, the bag can be withdrawn through the perineal wound without requiring additional surgery. After failed attempts at hemostasis with classic plugging and metal implants, some authors[29] successfully used plugging, applying traction with a breast implant that was inflated with 520 mL of saline solution placed in the presacral space and maintaining pressure on the SVP using the traction of the implant, which was connected to a 1-L bag of saline suspended at the end of the bed. Remzi *et al*[30] recommend plugging with a free graft of the rectus abdominis muscle measuring 4 cm × 2 cm × 1 cm sutured to the presacral tissue over the area of bleeding. The absence of necrosis and lack of abscesses with this technique are attributed to the hypervascularization of the presacral area and the revascularization of the graft. Civelek *et al*[31] applied bone wax (Bonewax®) directly to the presacral fascia and the periosteum and simultaneously used pelvic plugging. After the failure of classic pelvic plugging and

metal implants, Becker *et al*[32] recommend plugging the bleeding area directly with bone cement (polymethyl methacrylate). Moreover, the Bakri balloon, created specifically to be introduced into the uterine cavity to control bleeding[33], has been successfully used in 2 patients for the treatment of presacral bleeding after colorectal surgery[34].

Metal implants

Wang *et al*[4] first used pins in 1985 to control presacral bleeding. Since then, their implementation has been the subject of multiple communications, most of which reported good results[35-40]. However, pin placement can be technically difficult[22,37], especially in narrow pelvises[41], when the contour of the sacrum is not sufficiently smooth and regular or when osteoporotic disease is present in the bone[29,42]. Failure of the technique[14,32,41,43], development of a presacral hematoma, chronic pelvic pain, release, migration, and perianal extrusion of the implant[42], and the need for equipment that is not always routinely available in surgery[14,24] are complications and inconveniences associated with the implementation of this procedure. Its ineffectiveness for diffuse bleeding[44] has led to the development of other alternatives. Some authors[13,45] use the ProTack™ device to fix hemostatic sponges (Surgicel®) to the sacrum using helical titanium tacks. Wang *et al*[43] used saw-tooth staples of different sizes that fit into the gap between the staple and the sacrum, along with a spongy bone graft and a plate of Surgicel®. Jivapaisarnpong[46] reported the cessation of bleeding using vascular clips (Ligaclips®) in 3 patients in whom several other techniques, such as electrocauterization, coagulation with argon, indirect coagulation, and pelvic plugging, had failed.

Topical hemostatic agents

Topical hemostatic agents have been widely used, especially in cases of diffuse bleeding or when other methods have failed. For example, cyanoacrylate is a monomer that is purified by removing toxic products during its synthesis. Its contact with anionic substances such as blood causes it to polymerize into long chains that form a solid layer, resulting in hemostasis[47].

Losanoff *et al*[48] achieved hemostasis in 3 patients by evenly applying cyanoacrylate glue to the surface of a gelatin sponge measuring 3 cm × 2 cm; the sponge was then compressed for several minutes to ensure adequate contact with the presacral fascia and polymerization of the adhesive. Chen *et al*[49] used a combination of oxidized cellulose and cyanoacrylate. Specifically, they placed 2 to 5 pieces of 2 cm × 2 cm oxidized cellulose in a Kelly clamp and applied pressure to the injury for a few minutes. They then evenly applied 1 ml of cyanoacrylate to the cellulose surface and to the tissue surrounding the pieces of oxidized cellulose. Zhang *et al*[50] reported the control of bleeding in 5 patients by the application of pressure to the bleeding area with absorbable hemostatic gauze for 20-30 min. This gauze was similar to collagen and was created from cellulose that had been chemically treated and combined with alpha-cyanoacrylate as an adhesive. Karaman *et al*[51] achieved excellent results with the use of topical Ankaferd Blood Stopper® (ABS). ABS exhibits antihemorrhagic properties and is an extract of 5 medicinal plants that exert antithrombotic, antiplatelet, antioxidant, antiatherosclerotic, and antitumoral activities. Germanos *et al*[14] suggest that after several techniques have been tried and failed, presacral bleeding should be treated with direct hemostatic agents. Specifically, they used a gel formed by combining gelatin and thrombin (Floseal®) granules and an absorbable hemostatic agent, Surgicel®, prepared by the controlled oxidation of regenerated cellulose.

Direct suture

Some authors[14] report that clotting and direct suture are ineffective and should be avoided because they can exacerbate bleeding and cause significant blood loss[14]. Alternatively, Papalambros *et al*[52] report the potential benefits of temporarily clamping the infrarenal aorta, which hypothetically decreases blood flow in the vena cava and its tributaries and should reduce the hydrostatic pressure in the sacral plexus and bleeding. This approach would allow the identification of the point of bleeding and its suture. This procedure could be effective for treatment of type Ⅰ injuries described by Wang *et al*[4], which are easiest to treat and can be addressed with less bloody methods. However, in the opinion of other authors[23], this approach would be difficult to apply successfully to injuries of the basivertebral veins after retraction in the sacral periosteum. Ligature and circular suturing were described by Jiang *et al*[53] in 2013 as a method to control presacral venous bleeding. Once the bleeding points have been identified, the venous plexus is ligated and circularly sutured with 4/0 silk. The suture includes the presacral fascia, the presacral veins, and the deep connective tissue. Bleeding that continues after the first suture suggests that the blood originates from the communicating veins or the basivertebral veins, which necessitates a second or even a third suture. However, if bleeding originates from veins retracted in the bone, Jiang *et al*[53] recommend the implementation of a combination of techniques as more efficient than the use of a single method for the control of bleeding in this situation.

Direct or indirect electrocoagulation

Filippakis *et al*[54] controlled bleeding in 4 patients using electrocauterization in the spraying position at the bleeding points of the presacral fascia. Furthermore, Li *et al*[55] proposed direct bipolar coagulation as a simple and effective method for the management of presacral venous bleeding after demonstrating the

cessation of this type of bleeding in 7 patients. Kandeel *et al*[56] and Saurabh *et al*[57] reported 1 and 2 patients, respectively, in whom bleeding was controlled using argon coagulation.

Indirect monopolar electrocoagulation has been successfully used on a portion of an epiploic appendix by maintaining pressure on the bleeding area with a dissection clip[2,3]. Moreover, indirect electrocoagulation through a fragment of the anterior rectus abdominis muscle was described by Xu *et al*[58] and applied with success in 11 patients. The technique involves resecting a fragment of the anterior rectus abdominis muscle approximately 2 cm × 2 cm, placing it in a long dissection clip, applying pressure to the area of bleeding, and applying a monopolar current to induce clotting. Muscle is a soft tissue that contains approximately 75% water and is easily moldable to the bone surface. Water is an excellent conductor of energy due to the solutes dissolved in it. Thus, the implementation of electrocoagulation in muscle results in surgical smoke when the muscle is heated, and the cellular fluid is vaporized by the thermal action of the energy source. The temperature of the muscle gradually increases and reaches the boiling point after 90-120 s of application of monopolar current at maximum power. This temperature is the optimal coagulation point and ensures that the muscle adheres to the bone surface[23,58]. This method has been validated and used by other authors[22-24] with satisfactory results, in some cases after the failure of other alternatives. Specifically, it is a rapid, easily executed, and effective method that is usually free of intra- or post-operative complications and that can be used at several bleeding points. Furthermore, if the muscle does not adhere to the bone, the technique does not fail.

CONCLUSION

Sacral foramina that connect the internal venous plexus with the presacral venous plexus *via* the basivertebral veins are found at the levels of all vertebral bodies, and foramina of greater caliber are located at the level of S4-S5. Therefore, injury at that level presumably causes bleeding with greater flow that is difficult to control. The treatment algorithm we propose is based on the analysis of more than 50 articles presented in this review. This information can help the surgeon understand the physiopathology of and treatment strategies for presacral venous bleeding.

In our opinion and based on our experience with the occurrence of presacral bleeding during rectal surgery, indirect coagulation through the interposition of a fragment of the anterior rectus abdominis muscle is a very effective method when it is possible to identify the site of bleeding[23]. Other methods that have also proven effective are the use of topical hemostatic agents and the use of pins.

COMMENTS

Background

Presacral venous bleeding is a rare but potentially lethal complication of surgery for rectal cancer. Incorrect mobilization of the rectum that injures the presacral fascia or de-insertion of the anorectal fascia can cause bleeding in the sacral venous plexus. This bleeding can be very difficult to control at the level of the last sacral vertebrae due to injury to the large basivertebral veins.

Research frontiers

The present study aims to help surgeons understand the vascular anatomy of the presacral plexus, the pathophysiology of presacral bleeding, the factors influencing the flow of venous injury, the causes and types of damage, the incidence of presacral bleeding and the surgical strategies for treatment.

Innovations and breakthroughs

Due to the lack of cases published in the literature, there is no consensus on which is the best technique to use if there is presacral bleeding during mobilization in surgery for rectal cancer. This review may provide a tool to help surgeons make decisions regarding how to resolve this serious complication.

Applications

This review aims to provide a set of resources to resolve presacral bleeding.

Peer-review

This review will be helping surgeons understand the physiopathology of presacral bleeding and the surgical strategies for its treatment. It is really helpful.

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CASE REPORT

Gastrointestinal stromal tumor of the stomach with axillary lymph node metastasis: A case report

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Author contributions: Kubo N and Takeuchi N participated in the surgery of this case; Takeuchi N treated the patient after surgery; Kubo N drafted the manuscript and all authors read and approved the final manuscript.

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common type of gastrointestinal mesenchymal tumors, although metastasis to the perigastric lymph nodes is relatively rare, compared with liver or peritoneal metastasis. In this report, we describe a case of stomach GIST with a solitary simultaneous metastasis in the left axillary lymph node. A 68-year-old man was diagnosed with a large upper-stomach GIST, and computed tomography and positron emission tomography revealed masses in the left axilla and right mediastinum. We did not detect evidence of metastases to the liver, or other sites including the perigastric lymph nodes, although findings from the surgically resected axillary lymph nodes were compatible with GIST metastasis. Treatment using imatinib markedly reduced the gastric and mediastinal lesions, and this response persisted for 3 years. The patient subsequently experienced rapid growth of the gastric lesion without mediastinal or axilla recurrence, which required palliative surgery. Despite continuing medical treatment (sunitinib and regorafenib), the patient died of liver metastases 23 mo after the surgery. Based on our findings, it appears that the axillary lymph nodes can be a potential metastatic site for GIST metastasis.

Key words: Gastrointestinal stromal tumor; Axillary; Lymph node; Metastasis; Imatinib

Core tip: Gastrointestinal stromal tumors (GISTs) are the most common type of gastrointestinal mesenchymal tumors, although metastasis to the perigastric lymph nodes is relatively rare, compared to liver or peritoneal metastasis. In this report, we describe a case of stomach GIST with a solitary simultaneous metastasis in the left axillary lymph node. Based on our findings,

it appears the axillary lymph nodes can be a potential metastatic site of GIST metastasis.

Kubo N, Takeuchi N. Gastrointestinal stromal tumor of the stomach with axillary lymph node metastasis: A case report. *World J Gastroenterol* 2017; 23(9): 1720-1724 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1720.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1720

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common type of gastrointestinal mesenchymal tumor, although GIST only accounts for approximately 1% of gastric malignancies[1]. GISTs frequently metastasize to the liver or peritoneum, although lymph node metastasis is very rare[2], even to the perigastric area. Therefore, we report the first documented case of stomach GIST with simultaneous axillary lymph node metastasis.

CASE REPORT

A 68-year-old man was admitted to our hospital after complaining of anorexia and obvious weight loss. Gastroscopy revealed a large tumor with ulceration in the upper stomach body (Figure 1), and histological evaluation confirmed a diagnosis of a GIST (positive for c-kit and CD34). The mitotic index was 5/50 in high-power field and the MIB-1 labeling index was 10%. Computed tomography (CT) and positron emission tomography revealed a primary gastric tumor (diameter: 5 cm), a right mediastinal tumor (diameter: 2 cm), and a left axilla mass (diameter: 1 cm) (Figure 2). Based on these findings, we performed a complete gross excision of the left axilla mass. The specimen was 1.4 cm in diameter, and there was no extranodal extension, it exhibited monotonous spindle cells (Figure 3A-D) and was diagnosed as a metastasis of the GIST, because it exhibited positive immunohistochemical staining for c-kit (Figure 3E) and DOG1 (Figure 3F), the mitotic index was 15/50 in high-power field and the MIB-1 labeling index was 10%. Based on these findings, we started treatment using oral imatinib (400 mg/d), and in the next year, after starting imatinib, we followed up the patient every 3 mo by using CT and every 6 mo by using PET. The 6-mo follow-up revealed rapid response of the primary lesion and complete remission in the mediastinal lymph nodes. However, after 3 years of imatinib treatment, the primary gastric tumor exhibited rapid growth that resulted in obstructive symptoms and continuous bleeding, although we did not detect recurrence in the mediastinal and axillary lymph nodes. We performed total gastrectomy as palliative surgery, and all 13 resected perigastric lymph nodes were negative for metastasis; the mitotic index

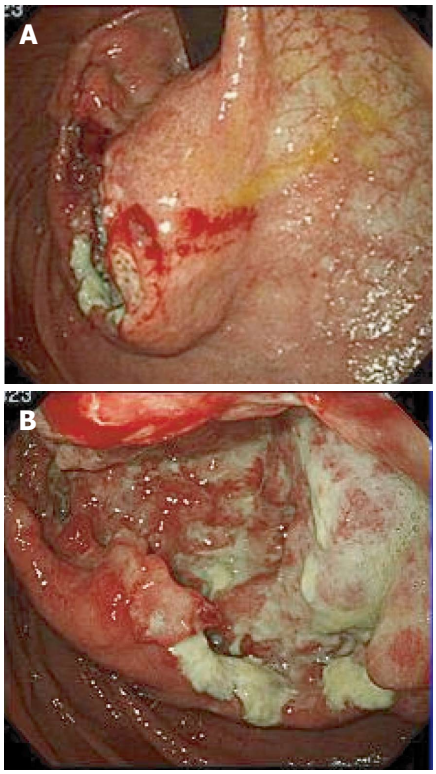

Figure 1 Gastroscopy revealed a large tumor with ulceration in the upper body of the stomach.

was 20/50 in high-power field and the MIB-1 labeling index was 30%. The patient subsequently recovered, and we started postoperative adjuvant treatment using imatinib. However, liver metastases appeared after 13 mo of treatment using imatinib, which we attempted to treat using regorafenib because gene sequence analysis of the tumor showed a KIT exon 11 mutation. The liver metastasis increased 2 mo later, and we started treatment with sunitinib. However, the patient developed renal dysfunction, and died of the liver metastasis 23 mo after the surgery with gastretomy.

DISCUSSION

GIST is the most common type of gastrointestinal mesenchymal tumor, and commonly exhibits mutations in the c-kit proto-oncogene[3]. GIST frequently metastasizes to the liver or peritoneum, although nodal metastasis is very rare[2]. Among 200 reported patients with digestive tract GIST, 94 patients exhibited metastasis, which included 61 liver metastases (65%), 20 peritoneal metastases (21%), and only 6 lymph node metastases (6%)[4]. To the best of our knowledge, there is only one documented case of distant lymph node metastasis from a stomach GIST, and that case involved inguinal lymph node metastasis[5]. Although a few cases of peripheral lymph nodes metastasis from GIST have been reported[6], the present case is the first documented case of a GIST presenting with solitary axillary lymph node metastasis.

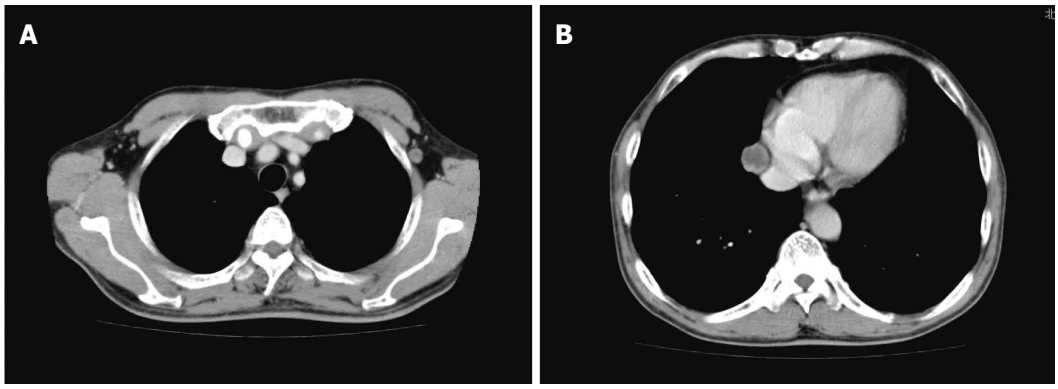

Figure 2 Computed tomography reveals a tumor in the left axilla (A, diameter: 1 cm) and a tumor in the right mediastinum (B, diameter: 2 cm).

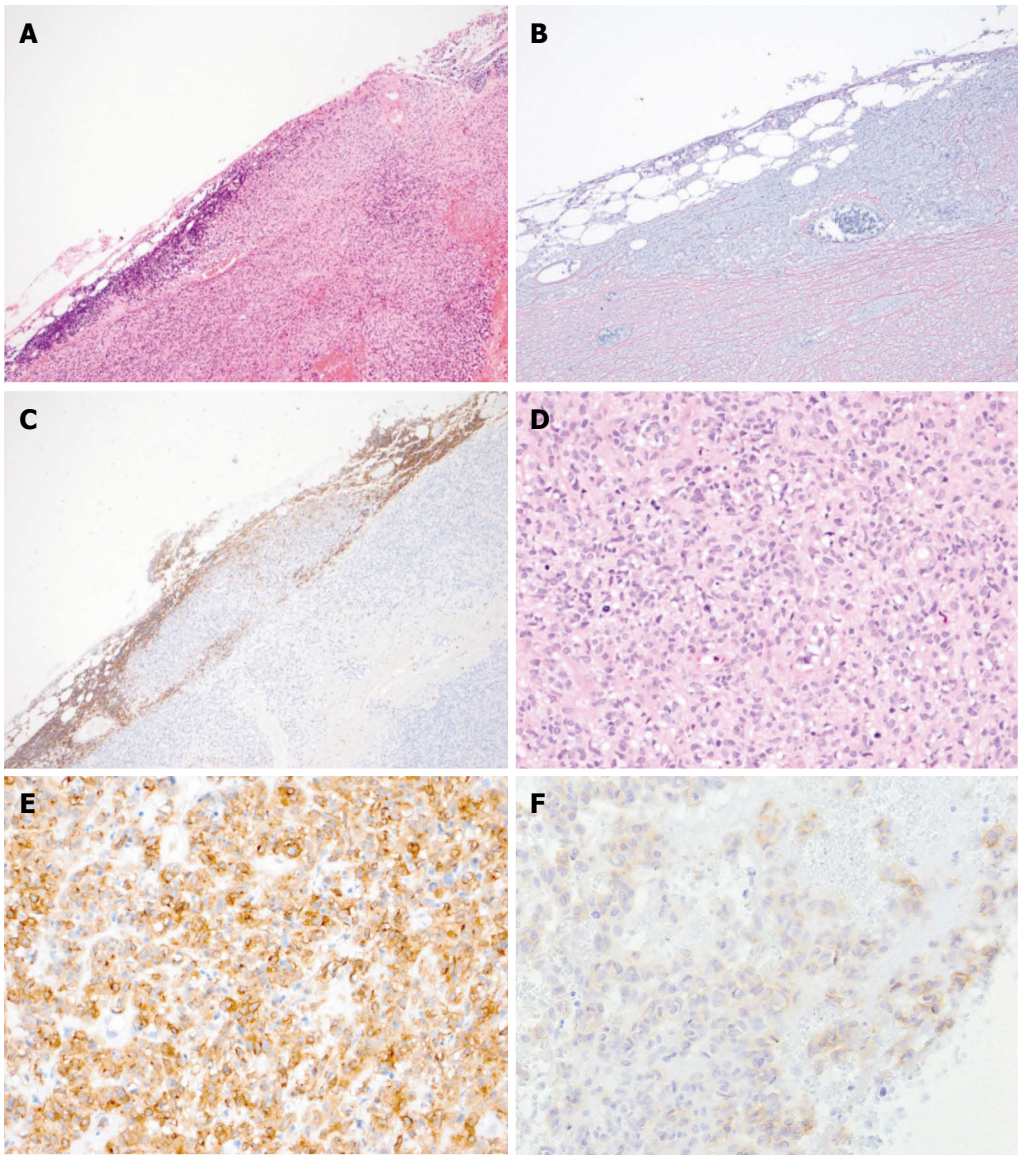

Figure 3 Pathological findings of the biopsied left axilla lymph node. Analysis of the tumor revealed tunicate formation and the survival of lymphoid tissue [hematoxylin and eosin staining(A), silver impregnation (B), and Leukocyte common antigen (C) (magnification × 40)]. The tumor exhibited monotonous spindle cells (D, hematoxylin and eosin staining), and the cells were positive for c-kit (E) and DOG1 (F, magnification × 100).

The three major routes of metastasis to the axillary lymph nodes are clearly documented in lung cancer[7,8]. The first route involves newly developed lymphatic channels that arise from the pleural lesions of adhesive lung tumors. The second route involves retrograde spread in the presence of supraclavicular

lymph node metastasis. The third route involves systemic axial lymph node metastasis. In this context, systemic metastases could be caused by the primary tumor invading nearby blood vessels and the subsequent dissemination of tumor cells into the venous system through the thoracic duct[9,10]. In the present case, the tumor was likely caused by systemic metastasis, as the recurrence was only detected in the right mediastinum, without lung or supraclavicular lymph node metastases.

Tumor size, location, mitotic rate, and C-KIT and PDGFRA genotype are the major determinants of the malignant potential of the tumor, and have significant impact on prognosis[11]. In the TNM (tumor-node-metastasis) system for GISTs, the presences of lymph node metastasis is classified as stage Ⅳ, which generally portends a poor prognosis, but cases with long-term survival have also been reported[5,12]. Valadao reported that lymph node metastasis is not related to poor prognosis; however, the study included a small number of patients[13]. Furthermore, it is unclear whether there is a difference of prognosis according to the site of lymph node metastasis, because reports of distant lymph node metastasis are very rare.

The appropriate therapy for GIST with distant metastasis remains controversial. Surgery is recommended if curative resection is possible, although imatinib therapy is occasionally selected in cases that have complications or may require expansive surgery. In the present case, we performed complete gross excision of the left axilla mass, and imaging revealed that only right mediastinum metastasis remained. However, we selected imatinib therapy, based on the tumor's stage and the patient's surgical stress. Six months of imatinib therapy markedly reduced the gastric lesion and the mediastinal lesion completely disappeared. In this context, resection of residual disease after imatinib pre-treatment is feasible in patients with metastatic GIST, even those with advanced hepatic and peritoneal metastasis[14], and surgery after achieving the best clinical response may be associated with a survival benefit (*vs* historical patients who were treated using imatinib alone)[15]. Therefore, we assumed that the distant metastasis had been controlled by imatinib and that curative resection was possible. However, the patient refused to undergo gastrectomy and elected to continue receiving imatinib. Unfortunately, the primary lesion exhibited rapid regrowth after 3 years of imatinib treatment without reappearance of the mediastinal lesion or other distinct metastases, which led to obstructive symptoms and continuous bleeding. At this point, we performed palliative total gastrectomy and provided ongoing medical treatment (sunitinib and regorafenib), although the patient ultimately died of liver metastases at 23 mo after the surgery. Nevertheless, the patient did not exhibit signs of distant lymph node metastasis.

In conclusion, the axillary lymph nodes can be a site of GIST metastasis, and imatinib chemotherapy may be useful for controlling distant lymph node metastasis from GIST. Although we performed a resection for the original lesion because the distant metastasis had been controlled by imatinib, the appropriate therapy for GIST with distant metastasis remains controversial. Further studies are needed to clarify the duration of chemotherapy and an appropriate surgical intervention that will be effective for treating distant lymph node metastasis.

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We thank Dr. M Fujiwara, Department of Pathology, Ina central hospital for his pathological diagnosis.

COMMENTS

Case characteristics

A 68-year-old man was admitted to our hospital after complaining of anorexia obvious weight loss.

Clinical diagnosis

The patient was diagnosed with malignancies in the stomach.

Differential diagnosis

Gastric cancer.

Imaging diagnosis

Gastroscopy revealed a large tumor with ulceration in the upper stomach body. Computed tomography and positron emission tomography revealed a primary gastric tumor and a left axilla mass.

Pathological diagnosis

Gastrointestinal stromal tumor of the stomach with axillary lymph node metastasis.

Treatment

Surgical resection and chemotherapy (imatinib, regorafenib and sunitinib).

Related reports

There is only one documented case of distant lymph node metastasis from a stomach gastrointestinal stromal tumor (GIST), and that case involved inguinal lymph node metastasis.

Term explanation

The axillary lymph nodes can be a site of GIST metastasis.

Experiences and lessons

Imatinib chemotherapy may be useful for controlling distant lymph node metastasis from GIST.

Peer-review

This is the interesting case report. The authors described lymph node metastasis of GIST is very rare; therefore, the appropriate therapy for GIST with lymph node metastasis remains controversial.

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CASE REPORT

Synchronous coexistence of liver metastases from cecal leiomyosarcoma and rectal adenocarcinoma: A case report

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Author contributions: Aoki H, Arata T, Utsumi M and Kunitomo T performed operations; Mushiake Y, Yasuhara I, Taniguchi F and Katsuda K collected and analyzed the data; Yamasaki R performed the histological examination; Tanakaya K and Takeuchi H supervised the research; Aoki H wrote this paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committees of Iwakuni Clinical Center.

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Abstract

Multiple liver tumors represent a challenging condition for abdominal surgeons both in the selection of technique and the rarity of diagnosis. There are no case reports on co-existence of liver metastases from both intestinal leiomyosarcoma and adenocarcinoma. The patient described in this report successfully underwent resection of both primary lesions and liver metastases in combination with chemotherapy. As for the leiomyosarcoma, the primary cecal lesion was revealed more than three years after the patient's first visit. Peritoneal, lymph-node, and lung recurrences were observed afterward, and thus surgeries on those regions were performed. Pathologically, the peritoneal and lung recurrences comprised leiomyosarcoma and the lymph-node recurrence was diagnosed as adenocarcinoma. Despite newly discovered multiple lung recurrences and regional lymph-node metastases, the patient lived a normal life for 73 mo after the initial operation based on multidisciplinary therapy. He ultimately died of liver failure due to invasive lymph-node recurrence from the rectal adenocarcinoma, in addition to multiple lung recurrences from the leiomyosarcoma. Hepatic recurrence did not occur in this patient's case, which appears to be one reason for his long-term survival.

Key words: Leiomyosarcoma; Chemotherapy; Multiple liver tumors; Liver metastasis; Adenocarcinoma

Core tip: There have been no case reports on co-existence of liver metastases from intestinal leiomyosarcoma and adenocarcinoma. This patient underwent resection of primary lesions and liver metastases in combination with chemotherapy. As for leiomyosarcoma, liver metastasis was discovered three years prior to discovery of the primary lesion. Peritoneal, lymph-node, and lung recurrences were discovered afterward, and therefore surgeries on those regions were performed. Despite newly discovered multiple lung recurrences and regional lymph-node metastases, the patient lived a normal life for 73 mo after the initial operation. He ultimately died of liver failure due to invasive lymph-node recurrence from the rectal adenocarcinoma.

Aoki H, Arata T, Utsumi M, Mushiake Y, Kunitomo T, Yasuhara I, Taniguchi F, Katsuda K, Tanakaya K, Takeuchi H, Yamasaki R. Synchronous coexistence of liver metastases from cecal leiomyosarcoma and rectal adenocarcinoma: A case report. *World J Gastroenterol* 2017; 23(9): 1725-1734 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i9/1725.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i9.1725

INTRODUCTION

Since the appearance of gastrointestinal stromal tumor as a distinctly defined entity, the diagnosis of intestinal leiomyosarcoma has not been common. Among the different types of this sarcoma, cecal leiomyosarcoma is extremely rare[1]. Hepatic leiomyosarcoma is also rare, particularly as a primary cancer[2]. Abdominal surgeons often confront multiple liver tumors, making treatment of such cases challenging. This is particularly the case if the diagnoses of hepatic tumors differ from each other. Until now, there had been no case reports regarding co-existence of liver metastases from a combination of intestinal leiomyosarcoma and adenocarcinoma. We hereby report on a patient who underwent successful treatment involving resection of both primary lesions and liver metastases along with chemotherapy. Peritoneal, lymph-node, and lung recurrences were observed afterward, and thus surgeries on those regions were also performed. Multiple lung recurrences and regional lymph-node metastases were newly discovered, but the patient could live a normal life for 73 mo after the initial surgery based on multidisciplinary therapy, which we will discuss in detail in this report.

CASE REPORT

A 61-year-old male visited our hospital with occult blood in stool and multiple liver tumors at an annual medical examination in October 2009. His past medical history involved only hypertension starting at the age of 57. At the examination, he underwent a colonoscopy examination, whereupon type 2 adenocarcinoma was discovered in the rectum. In addition, an abdominal computed tomography (CT) scan and gadoxetic acid enhanced magnetic resonance imaging revealed liver tumors in Segment 3, Segment 4, and Segment 8 (Figures 1 and 2). Anterior rectal resection with regional lymph-node dissection was carried out in December 2009. A pathological examination revealed extra serosal invasion by a moderately differentiated adenocarcinoma and metastases in eight of 14 resected lymph nodes.

Because genetic analysis confirmed wild-type *KRAS*, the patient received chemotherapy with modified FOLFOX6 plus bevacizumab after the rectal resection. Following four courses of FOLFOX, the S3 liver tumor disappeared and the S8 tumor decreased in size, but the S4 tumor was found to be enlarged. The patient then underwent seven courses of FOLFIRI plus bevacizumab followed by one course of irrinotecan plus cetuximab; as a result, the S4 and S8 tumors decreased in size (Figure 3). Positron emission tomography-CT after the chemotherapy series showed no significant uptake of fluorodeoxyglucose in the liver. The patient subsequently underwent a central bi-segmentectomy and a partial S3 resection in September 2010 (the second operation).

A pathological examination revealed fibrosis and calcification in the S3 and S8 tumors, with a few degenerated residual adenocarcinoma cells, which was compatible with rectal adenocarcinoma metastasis. In contrast, the S4 tumor consisted of irregular fascicles of spindle-shaped cells with eosinophilic cytoplasm and nuclear atypia. An immunohistochemical examination demonstrated that the S4 tumor cells were positive for α-smooth muscle actin and desmin, while negative for CD34, S-100, c-kit, and cytokeratin AE1/3 (Figure 4). Based on these findings, the S4 tumor was designated as leiomyosarcoma. The tumor grade was high according to the classification by Hajdu *et al*[3].

Histologically, chemotherapy had no apparent effect on this tumor. The patient underwent six courses of FOLFIRI plus bevacizumab as adjuvant chemotherapy after hepatic resection. Twenty-two months later (in July 2012), an abdominal CT scan was performed as part of an annual medical examination. A cecal tumor and lymph-node swelling around the common hepatic artery were discovered. Moreover, accumulation at both sites was discovered in a positron emission tomography (PET)-CT scan (Figure 5). The tumor had not been identified in a colonoscopy. Three months later, a type 2 tumor was discovered and a biopsy via colonoscopy revealed leiomyosarcoma. Ileocecal resection with lymph-node dissection and lymph-node sampling around the common hepatic artery were carried out in November 2012 (the third operation).

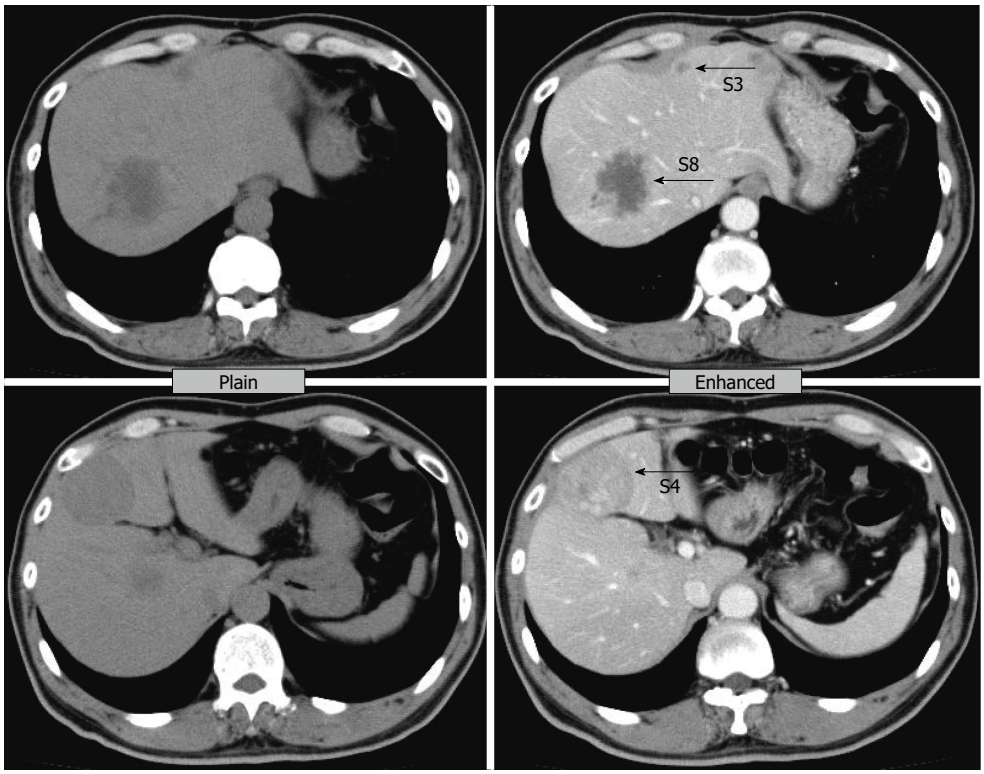

Figure 1 Computed tomography before treatment. An abdominal computed tomography scan revealed liver tumors in Segment 3, Segment 4, and Segment 8. The tumor in Segment 8 is hypodense with peripheral enhancement. The tumor in Segment 4 is a well-defined isodense tumor with homogeneous enhancement.

A pathological examination revealed cecal leiomyosarcoma with regional lymph-node metastases and metastasis from rectal adenocarcinoma in the lymph node around the common hepatic artery. Retrospectively, accumulation had been observed in the cecum in a PET-CT scan in September 2010, and a submucosal tumor had been suspected in a colonoscopy in July 2011. It is likely that rectal adenocarcinoma and cecal leiomyosarcoma existed synchronously from the beginning, and that both tumors had metastasized to the liver synchronously. The patient was then treated with nine courses of XELOX plus bevacizumab.

In a follow-up CT scan in June 2013, a tumor just below the peritoneum was discovered. Because this accumulation was identified in a PET-CT scan and no other accumulation was observed, extirpation of the tumor was carried out (the fourth operation). Pathological diagnosis was leiomyosarcoma of the omentum, compatible with recurrence (Figure 6). The patient was treated with XELOX plus bevacizumab following the surgery. Three months later, in a chest CT scan, two coin lesions were discovered in the left lung. There was no indication of accumulation at both lesions in a PET-CT scan, but lung metastases were strongly suspected. In October 2013 (46 mo after the first operation), partial resections of the left upper lobe and left lower lobe were performed (the fifth operation). Pathological diagnosis was metastatic leiomyosarcoma of the lung (Figure 7).

The patient was subsequently treated with XELOX plus bevacizumab again following surgery (the fifth and final operation). The hepatic hilum lymph node was found to be enlarged in July 2014, and multiple lung metastases were newly discovered in November 2014. Chemotherapy was changed to treatment consisting of CPT-11 plus cetuximab, due to neuropathy experienced by the patient. This clinical time course is demonstrated in Figure 8.

Although lymph-node and multiple lung metastases were present, the patient survived for more than six years after the initial operation. Since the left hepatic duct was constricted by lymph-node metastasis, a plastic stent was inserted in October 2015. The patient received chemotherapy with doxorubicin afterward as an outpatient but was hospitalized with cholangitis due to lymph-node metastatic recurrence in January 2016. The patient died from liver failure due to lymph-node invasion from rectal adenocarcinoma in March 2016 (76 mo after the initial operation). The extended treatment proved worthwhile, given that the patient could live a normal life for 73 mo after the initial operation with help from the multidisciplinary therapy we employed.

DISCUSSION

Hepatic resection for liver metastases from colorectal cancer or neuroendocrine tumor, in combination with chemotherapy, has been established as a safe and

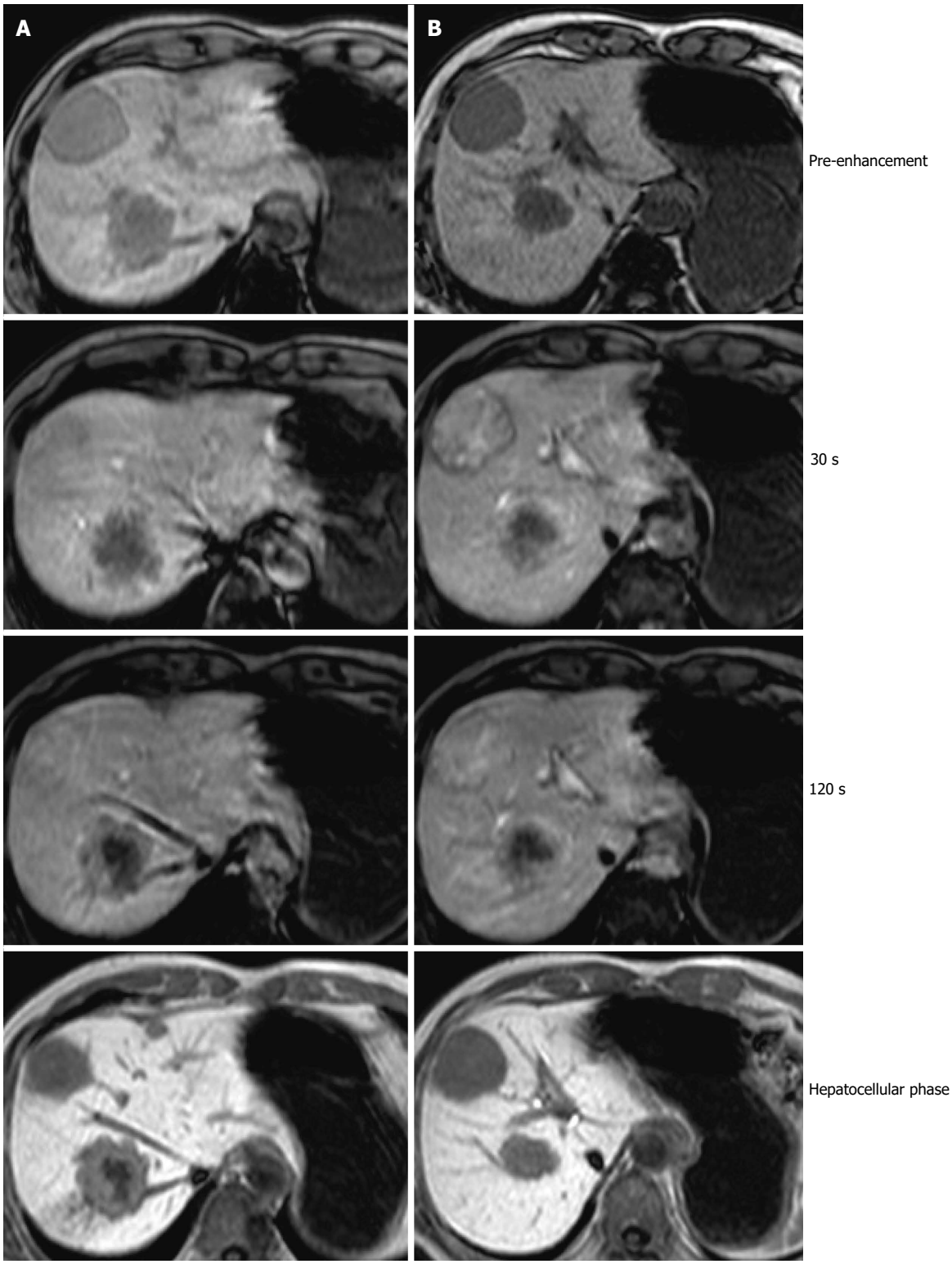

Figure 2 Ethoxibenzyl-magnetic resonance imaging before treatment. The tumors in Segments 3 and 8 showed gradual peripheral enhancement, while the tumor in Segment 4 showed heterogeneous enhancement and washout characteristics. A: S3 and S8 gradual peripheral enhancement; B: S4 heterogeneous enhancement.

standard treatment. The issue in question, however, is how to determine effective treatment for non-colorectal non-neuroendocrine liver metastases (NCNNLM). Hepatic metastasectomy had been thought ineffective for such cases. According to Gladdy's report of 353 patients with primary resectable leiomyosarcoma[4], recurrence occurred in 51% of abdominal and retroperitoneal leiomyosarcoma cases, including 29% of lung, 23% of liver, and 15% of other cases (brain and lymph nodes). Predictive factors for disease-free survival in patients with leiomyosarcoma were size and tumor grade. In addition, DeMatteo *et al*[5] reported

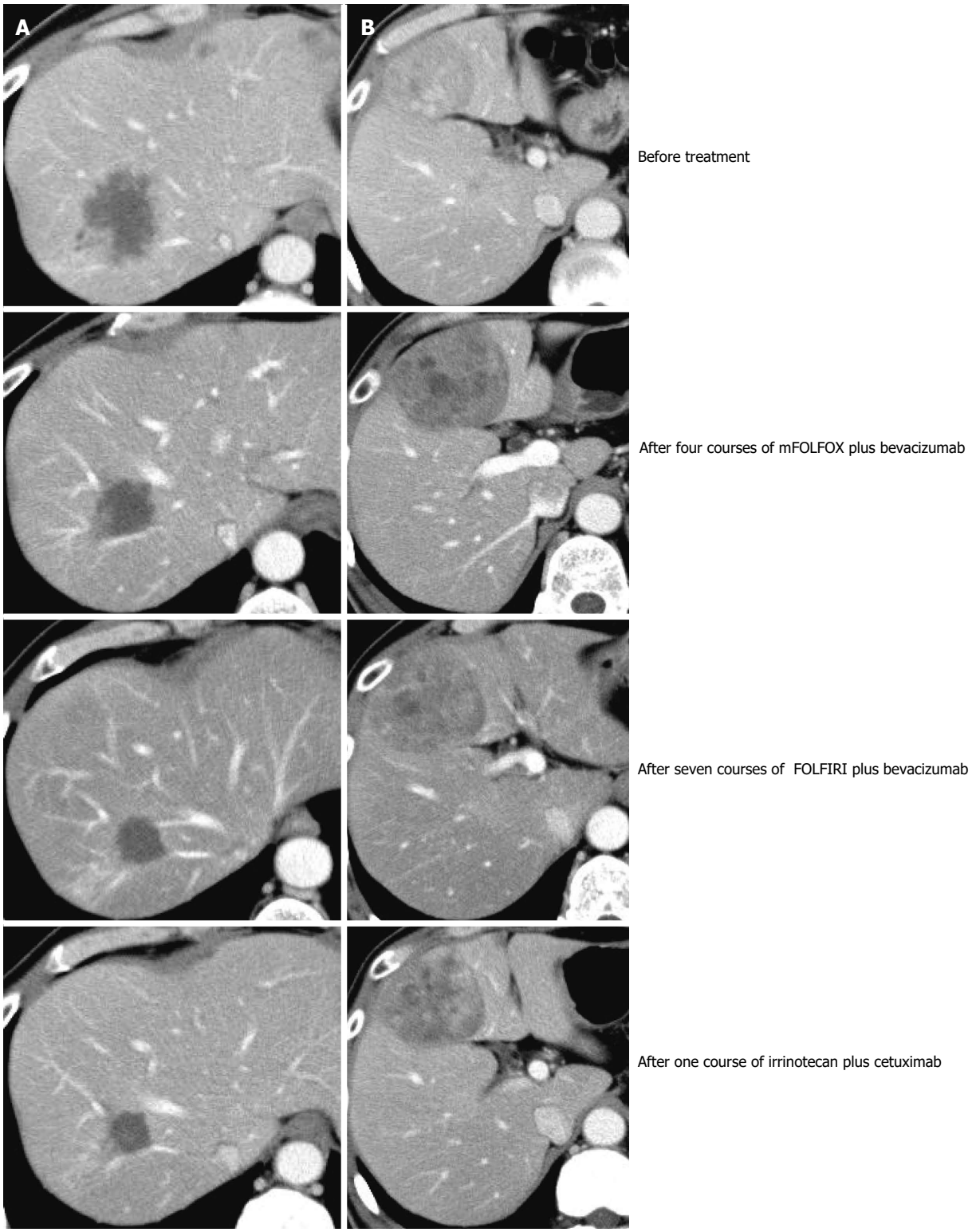

Figure 3 Changes in computed tomography images during treatment. The tumors in Segments 3 and 8 showed a gradual decrease in size, while the tumor in Segment 4 exhibited a one-time increase and then a decrease in size. A: S8 gradually decreased in size/S3 disappeared; B: S4 once increased then decreased in size.

that the rate of recurrence reached as high as 84% even after complete hepatic resection for sarcoma metastasis, although this definition of sarcoma includes gastrointestinal stromal tumors.

Ng *et al*[6] reviewed 191 cases of gastrointestinal leiomyosarcomas, of which colorectal leiomyosarcoma numbered 22 cases (12%). Without hepatectomy, the median survival time for patients with liver metastases from leiomyosarcoma was no more than 14 mo. Before the 21st century, metastases from leiomyosarcomas

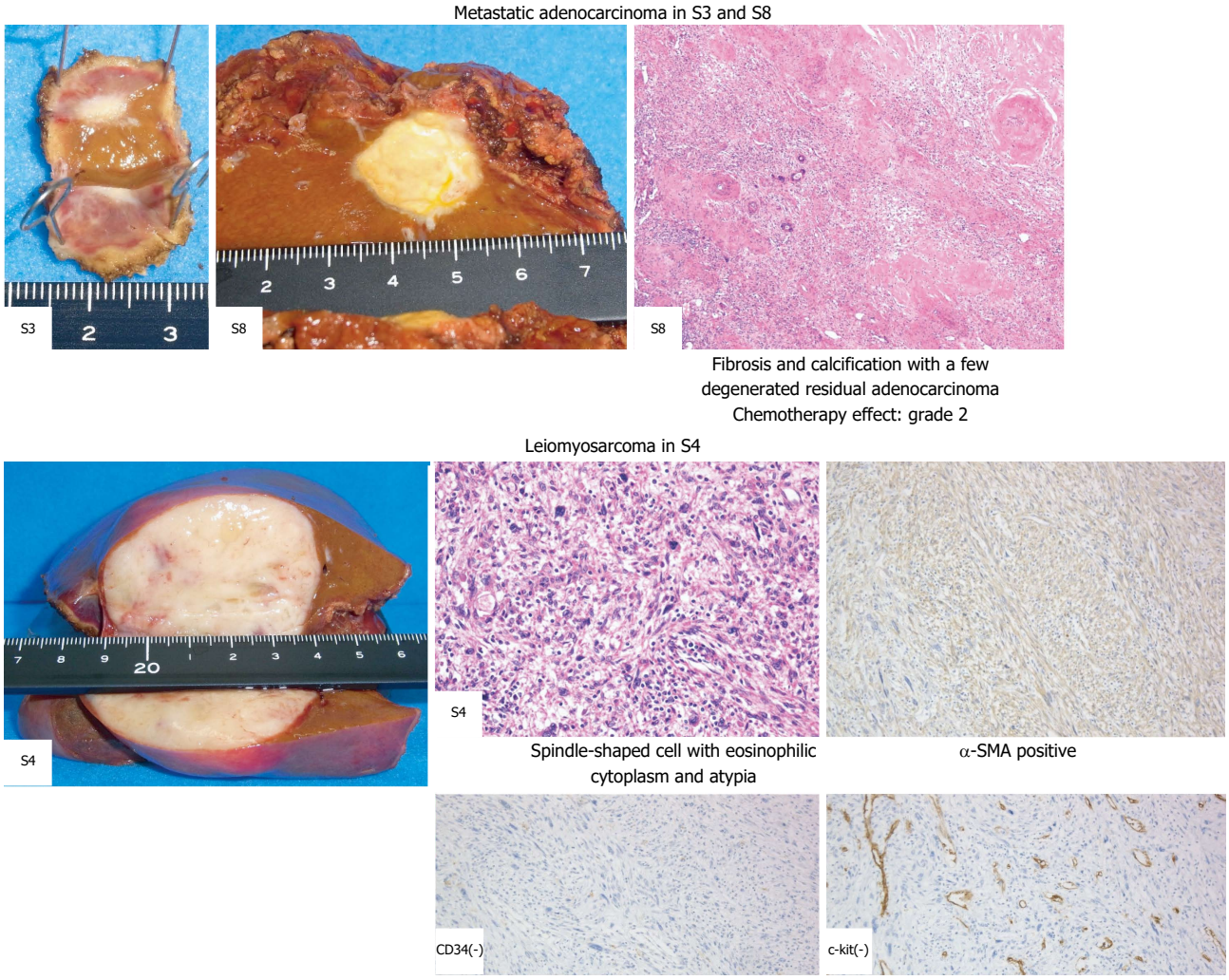

Figure 4 Pathological diagnoses of live lesions. The tumors in Segments 3 and 8 revealed fibrosis and calcification, with a few degenerated residual adenocarcinomas, while the tumor in Segment 4 consisted of irregular fascicles of spindle-shaped cells and was positive for SMA and negative for CD34 and c-kit. SMA: Smooth muscle actin.

were thought to not be sensitive to chemotherapy[7].

Lang *et al*[8] reported on 26 cases of hepatic metastases from leiomyosarcomas. Only one of these 26 cases (3.8%) originated from the colon. The median survival and five-year survival rate after R0 resection were 32 months and 20%, respectively. The presence of extrahepatic tumor growth should be regarded as a contraindication for liver resection only if an R0 resection does not appear possible. Marudanayagam *et al*[9] also emphasized the importance of R0 resection for hepatic metastasectomy of soft tissue sarcomas. Among soft tissue sarcomas, leiomyosarcoma was associated with poor prognosis.

Groeschl *et al*[10] reported on 420 patients who underwent hepatectomy for NCNNLM. The five-year survival rate in recent years was 32% after hepatectomy for liver metastases from sarcomas. Although the rate of recurrence after hepatectomy is as high as 66.5%, NCNNLM can be resected with reasonable survival outcomes when that surgery is appropriately selected as a treatment option. Prognostic factors for NCNNLM are tumor size (greater than 5 cm), lymphovascular invasion, and time-interval to liver metastasis of less than two years. Hepatic recurrence did not occur in the case we present here, which was one of the reasons for the patient's long-term survival.

We reported this patient's case as a primary hepatic leiomyosarcoma with liver metastasis of rectal cancer[2], before our finding of cecal leiomyosarcoma. The cecal leiomyosarcoma was discovered almost three years after the patient's first visit. From a pathological perspective, it was difficult to distinguish the site of primary lesion. Mourra *et al*[11] published a multi-institutional study on metastatic tumors in the colon and rectum. In that paper, only 35 of 10365 patients with colorectal malignancies (0.338%) were identified as having true metastases to the colon and rectum. Of those 35 metastatic colorectal tumors, leiomyosarcoma was identified in only two cases, with both tumors originating from soft tissue. This indicates only a small probability of the primary hepatic leiomyosarcoma metastasizing to the cecum.

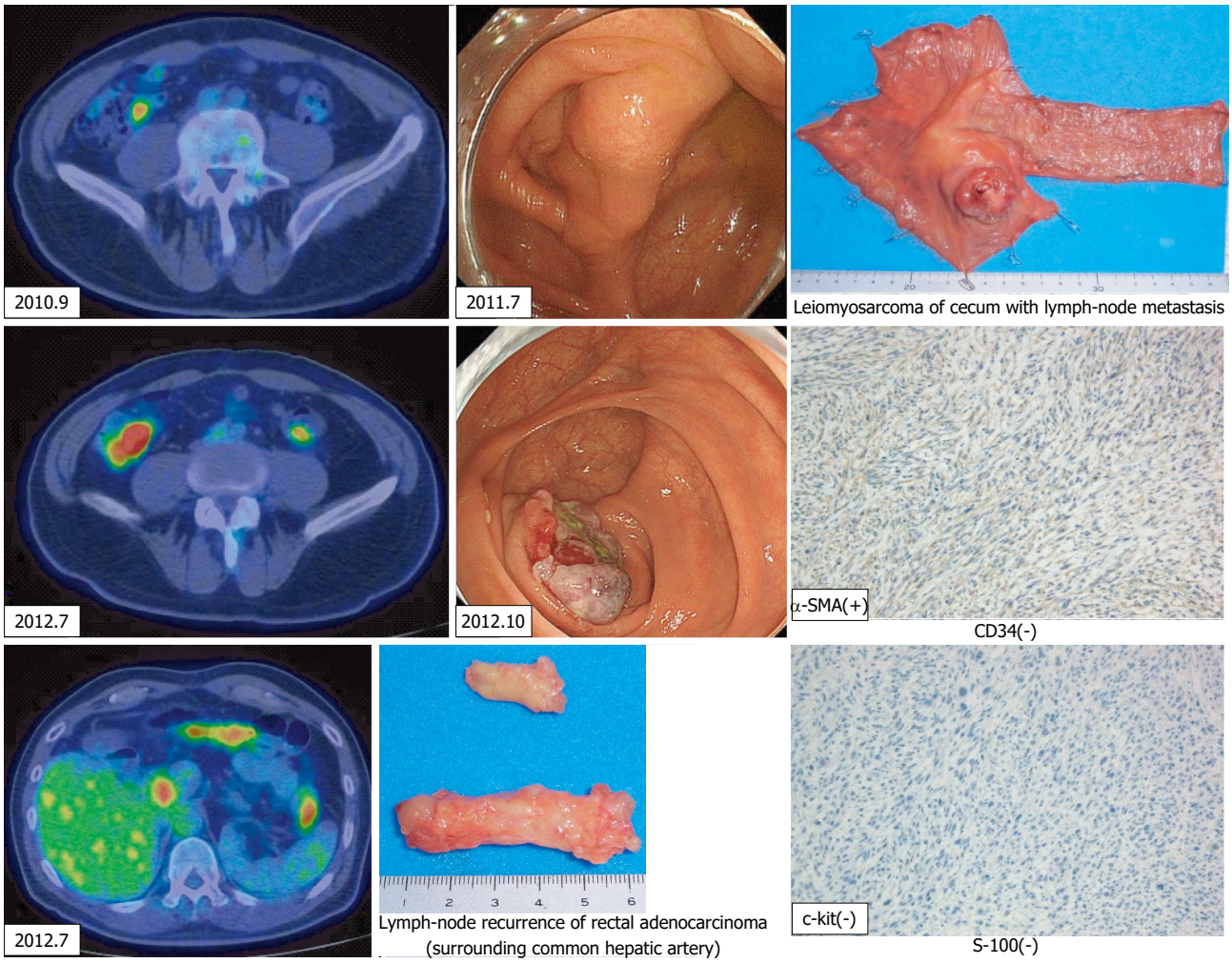

Figure 5 Cecal leiomyosarcoma and lymph-node recurrence. A cecal tumor and lymph-node swelling around the common hepatic artery were discovered in a positron emission tomography-computed tomography (PET-CT) scan. Retrospectively, accumulation had been observed in the cecum in a PET-CT scan in September 2010, and a submucosal tumor was suspected based on a colonoscopy taken in July 2011. SMA: Smooth muscle actin.

In contrast, hepatic metastasis is reported to occur in 20%-66.5% of patients with visceral or retroperitoneal sarcomas[9,10]. Moreover, all 35 patients had a history of metastatic disease in extragastrointestinal sites, with a mean disease-free interval of 10.6 years for sarcomas. We feel that these clinical characteristics were not compatible with our case, and for that reason we concluded that the cecum was the primary lesion site in the patient case we present here. We were aware that primary hepatic leiomyosarcoma is rare, and this patient's case has taught that its diagnosis should be made carefully only after long-term observation. However, rare are cases in which cecal leiomyosarcoma and rectal adenocarcinoma coexisted and metastasized to the liver. Hamai *et al*[12] reported a case of gastric adenocarcinoma with multiple liver tumors. After 14 mo of chemotherapy, the patient underwent total gastrectomy with partial liver resection, upon which the liver tumors were diagnosed pathologically as leiomyosarcomas. During adjuvant chemotherapy two years and five months after the first visit, a new hepatic tumor appeared and a tumor in the colon was discovered. The patient underwent partial colectomy and partial liver resection. The colon tumor and liver tumors were all immunohistochemically diagnosed as leiomyosarcomas. Seven months later, a third liver resection was carried out for the newly discovered liver tumors. Multiple liver and lung metastases eventually developed, and the patient died four years and 10 mo after the first visit. When a liver tumor is diagnosed as leiomyosarcoma, therefore, careful follow-up is needed to identify the primary site.

Leiomyosarcoma was for some time considered to be a relatively chemo-resistant sarcoma subtype. Recent data have demonstrated a reasonable response rate exhibited by some histological subtypes exposed to specific histology-tailored treatments with doxorubicin-containing chemotherapy[13]. Since without chemotherapy median survival is generally up to 12 mo[14], chemotherapy appears to be necessary for leiomyosarcoma treatment. However, the type of adjuvant therapy appropriate for leiomyosarcoma is an issue

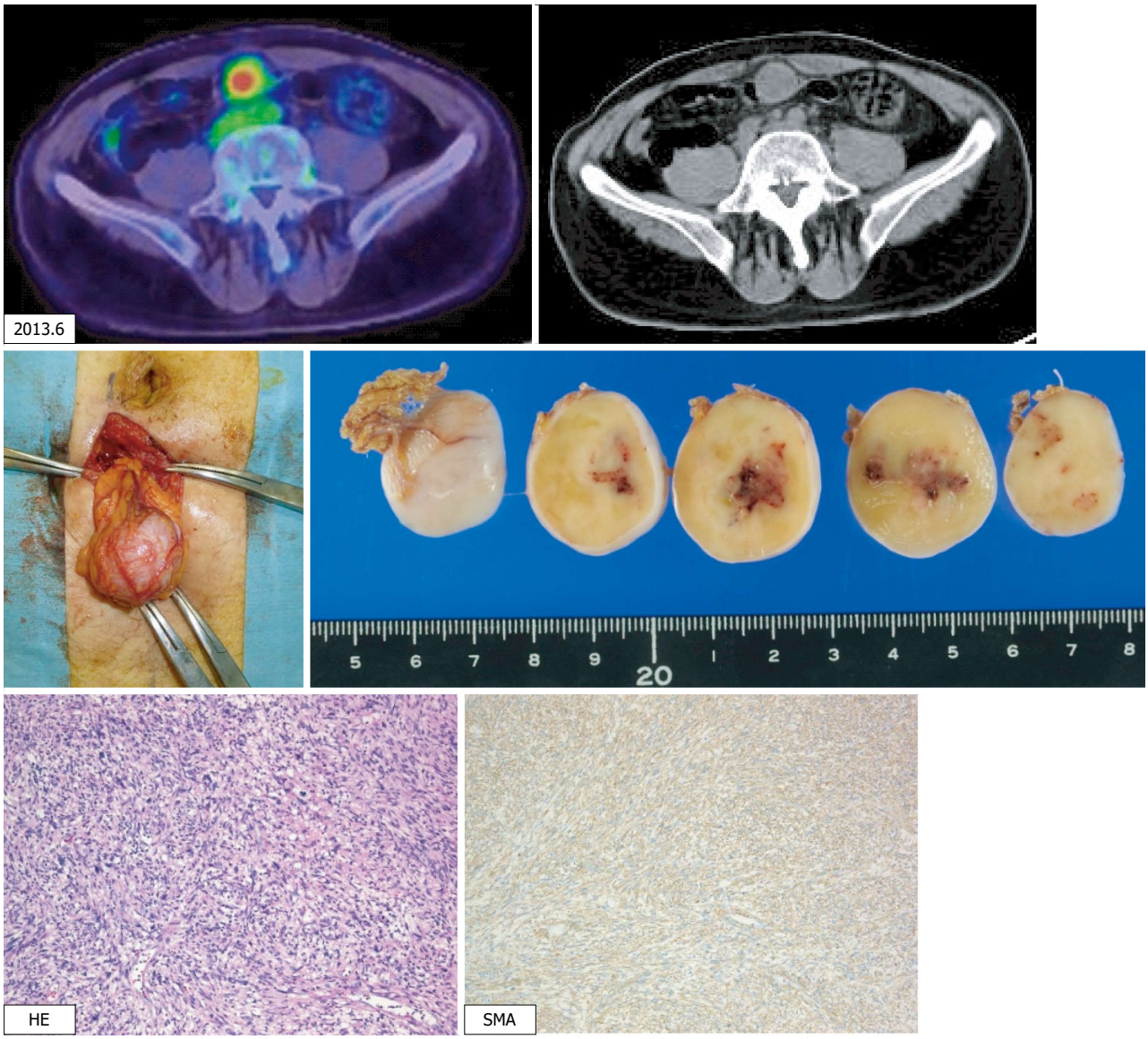

Figure 6 Peritoneal recurrences. A tumor just below the peritoneum was discovered. As the accumulation was recognized in a positron emission tomography-computed tomography scan and no other accumulation was observed, extirpation of the tumor was carried out. Pathological diagnosis was leiomyosarcoma of the omentum, compatible with recurrence. SMA: Smooth muscle actin.

requiring debate. Pazopanib was recently reported to be a feasible option for patients who had been heavily pretreated for metastatic sarcoma[15]. In that report, patients with leiomyosarcomas comprised the majority of long-term responders and survivors.

Both leiomyosarcoma and adenocarcinoma recurred after resection of primary lesions and hepatic metastases, making determination of a chemotherapy regimen for the patient in this report difficult. As we are familiar with adenocarcinomas and the distant lymph-node recurrence was adenocarcinoma pathologically, we chose a regimen mainly designed for colorectal adenocarcinoma. After peritoneal and lung tumors were diagnosed as leiomyosarcoma recurrence, we selected the treatment doxorubicin. At the patient's final hospitalization, lymph-node metastases to the hepatic hilum caused liver failure, which proved to be fatal, and chest X-rays showed numerous nodules in the lung field, which had caused a persistent cough in the patient. The former problem derived from adenocarcinoma and the latter from leiomyosarcoma. By that time, chemotherapy was no longer a treatment option.

The patient survived more than six years after initial diagnosis, even though he had both stage Ⅳ rectal cancer and cecal leiomyosarcoma with liver metastasis. As for long-term survival, Gladdy *et al*[4] reported on late disease-specific mortality in primary leiomyosarcoma. In that report, 6% of extremity and 9% of abdominal or retroperitoneal patients developed distant recurrence more than five years after the primary tumor diagnosis. The authors emphasized the need for long-term follow up.

The prognosis for patients with leiomyosarcoma might be prolonged in the future as chemotherapy

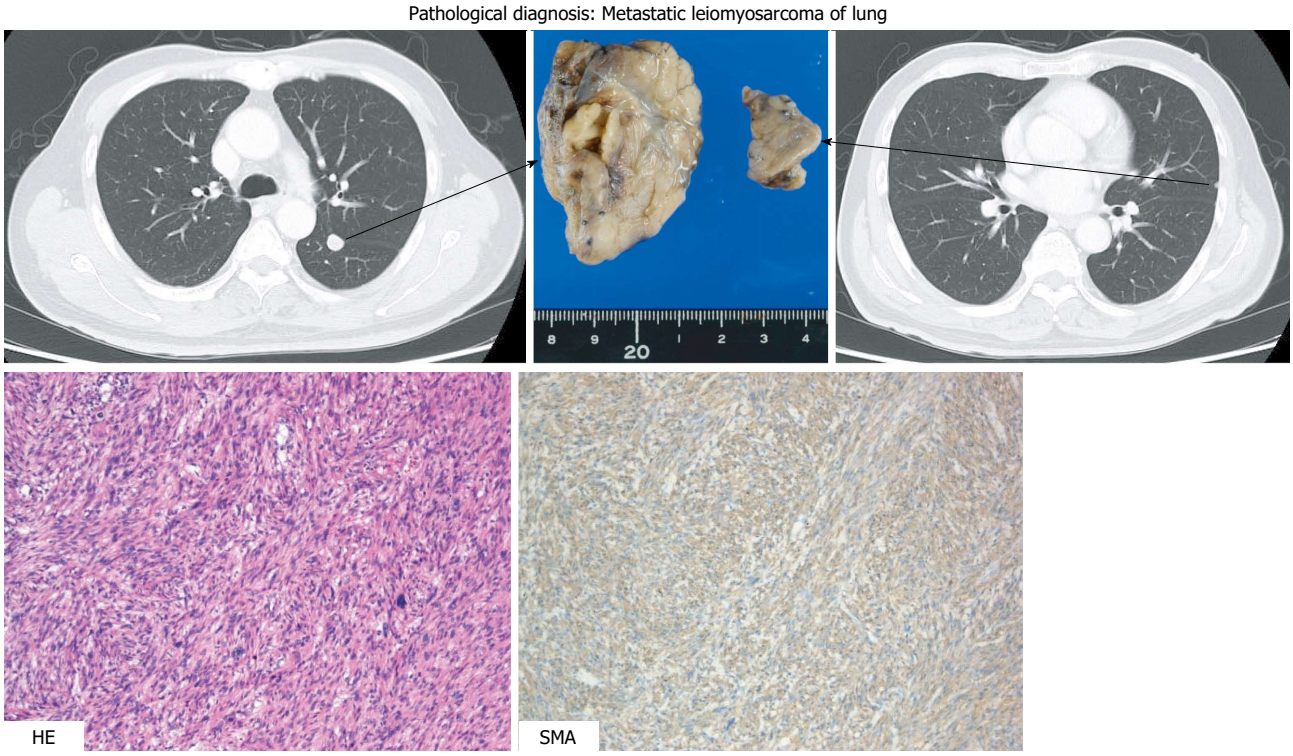

Figure 7 Lung recurrences. In a chest CT scan, two coin lesions were discovered in the left lung. As lung metastases were strongly suspected, partial resections of the left upper lobe and left lower lobe were performed. Pathological diagnosis was metastatic leiomyosarcoma of the lung.

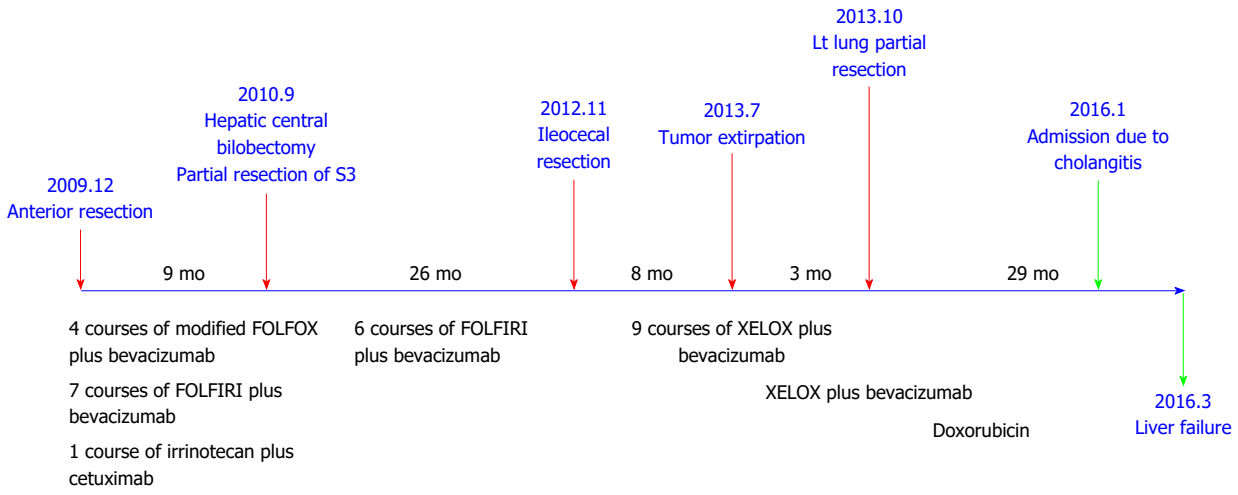

Figure 8 Clinical courses.

continues to advance. Long-term follow-up is important and should be considered.

This patient case was extremely rare for the points mentioned below: (1) cecal leiomyosarcoma and rectal adenocarcinoma coexisted; (2) both metastasized to the liver and were resected successfully; (3) primary colon leiomyosarcoma was diagnosed two years after resection of the hepatic metastatic lesion; and (4) long-term survival was attained based on multidisciplinary therapy.

COMMENTS

Case characteristics

A 61-year-old male patient with occult blood in stool and multiple liver tumors discovered at an annual medical examination.

Clinical diagnosis

Rectal adenocarcinoma with multiple liver metastases.

Differential diagnosis

The authors did not at first recognize a differential diagnosis.

Laboratory diagnosis
All lab measurements were within normal ranges except for slightly elevated serum carcino-embryonic antigen.

Imaging diagnosis
An abdominal computed tomography scan and gadoxetic acid enhanced magnetic resonance imaging revealed liver tumors in Segment 3, Segment 4, and Segment 8.

Pathological diagnosis
The patient had synchronous liver metastases from both cecal leiomyosarcoma and rectal adenocarcinoma.

Treatment
The patient successfully underwent resection of both primary lesions and liver metastases in combination with chemotherapy.

Related reports
There have been no case reports on co-existence of liver metastases from both cecal leiomyosarcoma and rectal adenocarcinoma. Only one report was published regarding a gastric adenocarcinoma with multiple liver tumors, which were diagnosed pathologically as leiomyosarcoma after gastrectomy and hepatectomy. Fifteen months later, a tumor in the colon was discovered, after which a partial colectomy was carried out. The colon tumor was immunohistochemically diagnosed as leiomyosarcoma.

Experiences and lessons
Treatment for multiple liver tumors is challenging, particularly if the diagnoses of hepatic tumors differ from each other.

Peer-review
The main issue regarding diagnosis is whether the hepatic leiomyosarcoma is a primary lesion or a metastasis from the cecum. From a pathological perspective, it is difficult to distinguish which of the sites is a primary lesion. The authors therefore went about trying to make a clinical determination.

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